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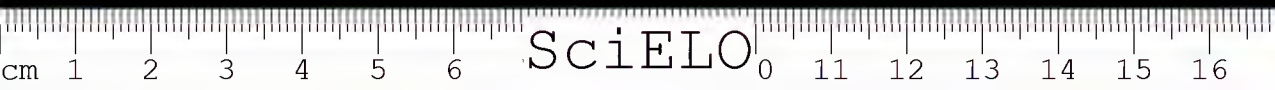
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MEMÓRIAS DO INSTITUTO BUTANTAN

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SIMPÓSIO SOBRE "TECNOLOGIA DO DNA RECOMBINANTE NA PRODUÇÃO DE VACINAS E NO DIAGNÓSTICO DE DOENÇAS INFECCIOSAS"

6-7 fevereiro 1991

Instituto Butantan
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1991

INSTITUTO BUTANTAN 1901—1991

SIMPÓSIO SOBRE "TECNOLOGIA DO DNA RECOMBINANTE NA PRODUÇÃO DE VACINAS E NO DIAGNÓSTICO DE DOENÇAS INFECCIOSAS"

SYMPOSIUM ON "RECOMBINANT DNA TECHNOLOGY IN THE PRODUCTION OF VACCINES AND THE DIAGNOSIS OF INFECTIOUS DISEASES"

Evento comemorativo dos 90 anos de fundação do Instituto Butantan

Comissão Organizadora do Simpósio

Eva Maria A. Kelen (Coordenadora)
Harumi Takehara
Isaías Raw
Willy Beçak
Wilmar Dias da Silva

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Fundação de Amparo à Pesquisa do
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Symposium on "Technology of recombinant-DNA in the production of vaccines and in the diagnosis of infectious diseases".

Instituto Butantan, February 6-7, 1991

PROGRAM

Febr 6, 1991 - Wednesday

Morning session: Chairperson: Isaías Raw (I. Butantan, S. Paulo, Brazil)

08:30-08:45 Opening of the Symposium by the Director of the Instituto Butantan
08:45-09:45 "Genetic engineered vaccines against rabies and rabies-related viruses: a review".
Noel Tordo (I. Pasteur, Paris, France)
09:45-10:00 Coffee Break
10:00-11:00 "Engineering bacterial toxins for the development of new vaccines".
R. Rappuoli (Lab. Sclavo, Siena, Italy)
11:00-12:00 Discussion
12:00-14:00 Lunch

Afternoon session: Chairperson: Beatriz Lieblich Fernandes (University of São Paulo, S. Paulo, Brazil)

14:00-14:30 "Insertions of heterologous epitopes in *Salmonella* flagellin".
Salete Newton (University of S. Paulo, Brazil)
14:30-15:00 "Cloning and characterization of *Tripanosoma cruzi* antigens and their use in the diagnosis of Chagas disease".
Samuel Goldenberg (Fiocruz, Rio de Janeiro, Brazil)
15:00-15:20 Coffee Break
15:20-15:50 "New development in diagnosis and control of animal health"
Ingrid E. Bergman (Pan Am. Center for Foot-and-Mouth Disease, Rio de Janeiro, Brazil)
15:50-16:50 Discussion

Febr 7, 1991 - Thursday

Morning session: Chairperson: Wilmar Dias da Silva (I. Butantan)

08:30-09:30 "Molecular epidemiology of rabies virus throughout the world; evaluation of the relationship between wild and vaccinal rabies strains".
Noel Tordo (I. Pasteur, Paris, France)
09:30-09:45 Coffee Break
09:45-10:45 Recombinant vaccines against whooping cough: development and clinical experience".
R. Rappuoli (Lab. Sclavo, Siena, Italy)
10:45-11:45 Discussion

Post-Symposium activity

Febr 8, 1991 - Friday

Morning session: Coordinator: Willy Beçak (I. Butantan)

09:00-10:00 "Perspectives of anti-meningitis B vaccine production".
Carl E. Frash (Bethesda, Maryland, USA)
10:00-10:15 Coffee Break
10:15-11:15 Discussion

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SIMPÓSIO "TECNOLOGIA DO DNA RECOMBINANTE NA PRODUÇÃO DE VACINAS E NO DIAGNÓSTICO DE DOENÇAS INFECCIOSAS"

O Instituto Butantan comemora este ano 90 anos de existência. Foi fundado para atender problemas prementes de saúde pública, no caso um surto epidêmico de peste bubônica que assolava a cidade de Santos.

No decorrer dessas nove décadas a instituição cresceu e expandiu-se. Atualmente, as suas atividades e seus programas podem ser resumidos no desenvolvimento do tripé ciência, tecnologia e educação.

A ampla gama de soros e vacinas produzidos, num total de 27 tipos diferentes, garantem ao Instituto Butantan a posição de maior instituto de imunobiológicos da América do Sul. No entanto, a sua filosofia não é o de uma mera fábrica de imunobiológicos, mas, principalmente, o de gerar, aperfeiçoar e transferir tecnologia moderna, nessa área, aos órgãos participantes dos programas de saúde pública.

Como substrato a essa tecnologia, desenvolve o Instituto Butantan ciência básica nos vários campos relacionados a medicina e a biologia. Ênfase grande tem sido dada a programas interdisciplinares que abrangem as novas áreas de conhecimentos que utilizam as modernas técnicas de biologia molecular e de engenharia genética.

É, portanto, não só oportuno, mas altamente significativo a inclusão no programa de comemorações do aniversário do Instituto Butantan, do simpósio sobre "Tecnologia do DNA Recombinante na produção de Vacinas e no Diagnóstico de Doenças Infecciosas". Os conhecimentos gerados, as descobertas, as vacinas e as proteínas purificadas obtidas por engenharia genética, os tratamentos preventivos e sintomáticos de doenças, terão nos próximos anos um impacto formidável na medicina e saúde pública. A política de saúde projetada para o futuro implica obrigatoriamente na existência de instituições de pesquisa e desenvolvimento que dominem as novas tecnologias, que podem ser definidas como biotecnologia, na acepção moderna da palavra.

No Instituto Butantan, o desenvolvimento tecnológico surge, naturalmente, como interface entre a pesquisa básica e a produção de imunobiológicos. É nesse segmento que a tecnologia do DNA recombinante, que é o tema central desse simpósio e no qual contamos com a participação de renomados cientistas nacionais e estrangeiros, tem grande importância.

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É imprescindível para a geração dessa tecnologia em níveis e volume adequados, a formação de uma massa crítica de pesquisadores familiarizados com esses conhecimentos. Nesse sentido o Instituto Butantan tem investido na constante reciclagem dos seus pesquisadores e técnicos e nos recém criados cursos de especialização e curso de pós-graduação em biotecnologia. Simpósios como o que iniciamos hoje fazem parte desse programa estratégico de formação de pessoal.

Damos as boas vindas a todos os participantes e convidados desse Simpósio e em especial aos cientistas de outras instituições do Brasil e do exterior, que estão aqui conosco para apresentar suas contribuições e participar dos debates. É necessária uma cooperação maior entre os cientistas de países de economia menos e mais adiantada, para a solução dos graves problemas de saúde pública, que representam um desafio, cuja solução é de nossa responsabilidade.

É para mim, uma honra e satisfação fazer a abertura desse importante conclave científico, augurando sucesso.

WILLY BEÇAK
Diretor Geral
Instituto Butantan

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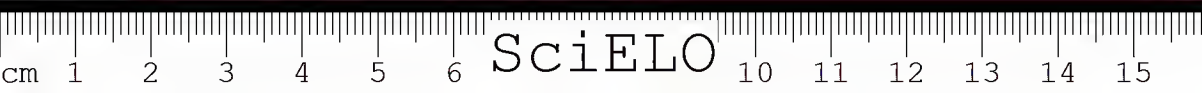
OPENING REMARKS

SYMPOSIUM "RECOMBINANT DNA TECHNOLOGY IN THE PRODUCTION OF VACCINES AND THE DIAGNOSIS OF INFECTIOUS DISEASES"

The Instituto Butantan commemorates this year 90 years of existence. It was founded in order to attend urgent problems of the public health, in particular, an epidemic outbreak of bubonic plague that devastated the city of Santos.

In the course of these nine decades, the Institution grew and developed. Its present activities and programmes can be summarized as: development of science, technology and education.

The wide range of 27 different sera and vaccines produced, assures to the Instituto Butantan the position of the largest plant of immunobiologicals of South America. However, its phylosophy is not to be a simple factory of immunobiologicals, but mainly to generate, improve and transfer modern technology within this area to the participating organs of the Public Health programmes.

As substrate of this technology, the Instituto Butantan develops basic science in the various fields related to Medicine and Biology. Emphasis is given to interdisciplinary programs which include new areas of knowledge using modern techniques of molecular biology and genetic engineering.

Therefore it is not only opportune, but highly significant the inclusion in the program of commemorations of the Instituto Butantan's anniversary, the Symposium on "Recombinant DNA Technology in the production of vaccines and diagnosis of infectious diseases". The generated knowledge, the vaccines and the purified proteins obtained by genetic engineering, the preventive and symptomatic modern treatment of diseases will play an important role in medicine and public health in the coming years. The future health policy has necessarily, to imply that institutions dedicated to research and scientific development should dominate the new technologies, known as Biotechnology in the modern concept of the term.

In the Instituto Butantan, the technological development appears naturally, as an interphase of basic research and production of immunobiologicals. In this segment, the recombinant DNA, central topic of this symposium in which renowned national and foreign scientists will participate, is very important. To enhance the use of this technology at adequate level and volume we have to assemble a critical mass of well trained sci-

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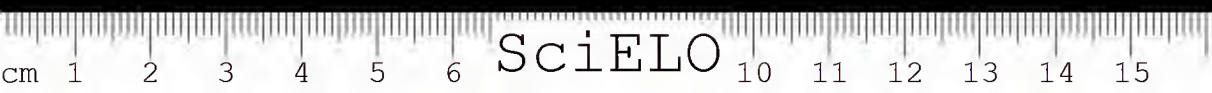
entists. In this sense, the Institute is investing in recycling its staff and in organizing new courses of specialization and postgraduation mainly in biotechnology. Symposia as the one we are opening today are part of this strategic program.

We wellcome all participants and invited guests of this Symposium, in particular the scientists from our country and from abroad, who accepted our invitation to present their contributions and to participate in the discussions. A greater cooperation is necessary among scientists from different countries to solve the serious problems of public health, that are a challenge, the sollution of which is our responsability.

It is a honour and satisfaction for me to open this important scientific meeting, wishing it to be a great success.

W. BEÇAK
Director
INSTITUTO BUTANTAN

1 – Left to right: Ingrid E. Bergmann, Pan American Center of Foot-and-Mouth Disease, Rio de Janeiro, Brazil; Rino Rappuoli, Sclavo Research Center, Siena, Italy; Salete Newton, Dept. Microbiology, University of S. Paulo, Brazil; Noël Tordo, Dept. Virology, Institut Pasteur, Paris, France.

2 – Samuel Goldenberg, Dept. Biochemistry and Molecular Biology, Fundação Oswado Cruz, Rio de Janeiro, Brazil.

3 – Carl E. Frasch, Center for Biologics Evaluation and Research, Bethesda, Maryland, USA.

4 – Willy Beçak, Director of the Instituto Butantan, S. Paulo, Brazil.

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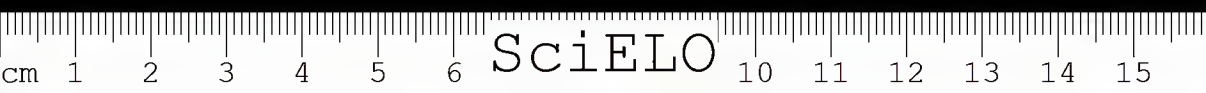
ENGINEERING BACTERIAL TOXINS FOR THE DEVELOPMENT OF NEW VACCINES

M.G. Pizza, M. Domenighini, L. Nencioni, A. Podda, R. Vanni, S. Silvestri, R. Rappuoli, Sclavo Research Centre, Siena, Italy

BACTERIAL ADP-RIBOSYLATING TOXINS

ADP-ribosylating bacterial toxins are proteins, produced by pathogenic bacteria, which are usually released into the extracellular medium and cause disease by killing or altering the metabolism of eukaryotic cells. The toxins are usually composed of two functionally distinct domains: a toxic moiety and a vector which have been called domains A and B, respectively. The vector (B) binds the receptors on the surface of eukaryotic cells and delivers the toxic part (A) across the membrane of eukaryotic cells so that it can reach its target proteins[1].

While the properties and the complexity of the vector (B) differ from toxin to toxin and often also within the same family of toxins, all of the A domains have a common mechanism of action: they are enzymes which ADP-ribosylate eukaryotic target proteins which control crucial circuits of eukaryotic cells, such as protein synthesis, transmembrane signaling, oncogenesis, cytoskeleton structure. The target proteins also have a common feature and a common structure: they are GTP-binding proteins. The only ADP-ribosylating toxins characterized both in terms of protein and genetic structure are diphtheria toxin (DT), Pseudomonas exotoxin A (PAETA), pertussis toxin (PT), cholera toxin (CT) and the *E.coli* heat-labile toxin (LT). The main properties of these toxin s are summarized in Table I.

THE ACTIVE SITE OF ADP-RIBOSYLATING TOXINS HAS A COMMON STRUCTURE

The initial comparison of the aminoacid sequences of DT and PAETA did not show sequence homology[2]. However, when by photoaffinity labeling Carroll and Collier showed that Glu148 of diphtheria toxin is functionally equivalent to Glu553 of Pseudo-

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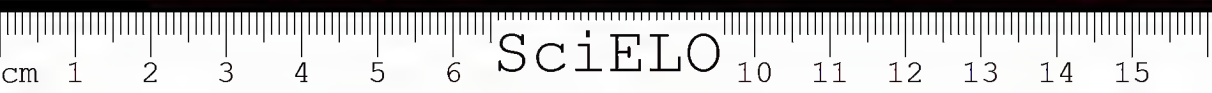


TABLE I

Bacterial toxin	Acceptor protein	Acceptor aminoacid	Effect on eukaryotic cells	Primary	Eukaryotic cell structure receptor
Diphtheria toxin	Elongation factor-2	Diphtamide 715	Inhibition of protein synthesis	Known	14.5 Kd protein?
Pseudomonas ETA	Elongation factor-2	Diphtamide 715	Inhibition of protein synthesis	Known	Not known
Pertussis toxin	Gi, Go, T	Cys 352	Alteration of transmembrane signal transduction	Known	160 Kd glycoprotein in CHO cells
Cholera toxin	Gs, T (Gi and Go	Arg 201	"	"	Ganglioside GM1>GDb1
E. coli LT1	"	Arg 201	"	"	Ganglioside GM1>GDb1>GM2

monas exotoxin A[3] they realigned the aminoacid sequences using this reference point and found a strong homology between the two toxins[4]. Regions of strong homology were also found between pertussis and cholera toxins[5, 6]. The two groups of toxins (which have a different target) did not show any aminoacid homology.

In the meantime, by chemical modification, photoaffinity labeling and site-directed mutagenesis, a number of aminoacids were identified which are essential for the catalytic activity of the different toxins[2]. Remarkably, these aminoacids were found to be conserved in all enzymes (Fig. 1).

The availability of the three-dimensional structure of PAETA, determined by X-ray crystallography by Allured et al.[7] provided the template for a computer-based molecular modeling of DT, PT and CT. Using the coordinates of ETA we were able to predict the majority of the structure of DT and the structure of the active sites of PT and CT. As shown in Fig. 2, the active site of the four toxins which contain all aminoacids identified in Fig. 1 share a common structure.

SITE-DIRECTED MUTAGENESIS OF THE ACTIVE SITE AND CONSTRUCTION OF NON TOXIC MOLECULES

The identification of the aminoacids which are in the active site of the ADP-ribosylating toxins, provided the rational basis for the mutagenesis of their genes in order to obtain non toxic molecules to be used in vaccines. A number of non toxic molecules have been obtained from diphtheria toxin, pseudomonas exotoxin A and pertussis toxin[8]. In all cases, the substitution of the glutamic acid (residue 6 in Figs. 1 and 2) or its deletion was the mutation most effective in reducing the toxicity of the molecules.

Among the many non toxic derivatives obtained, the one which has been studied in more detail is PT-9K/129G, a non toxic mutant of pertussis toxin which is being actively tested in clinical trials as a new vaccine against whooping cough. This mutant contains

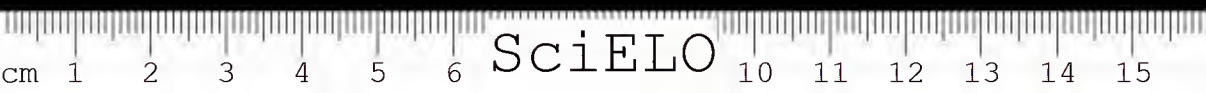
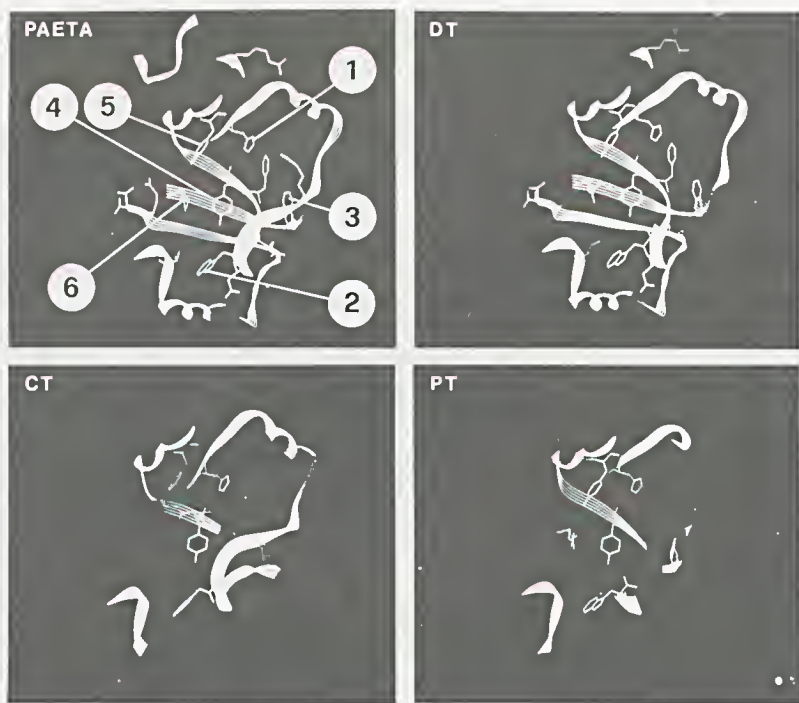



Figure 1 - Homologies between the three regions forming the active site of Pseudomonas exotoxin A (PAETA) and corresponding regions of diphtheria toxin (DT) pertussis toxin (PT), cholera toxin (CT) and E. coli LT toxin (LT).

Figure 2 - Structure of the active sites of PAETA, DT, CT and PT, obtained using the coordinates of PAETA. The numbers identify the aminoacids shown in Fig. 1.

two aminoacid substitutions (Arg9 —> Lys9 and Glu129 —> Gly) which make this molecule absolutely non toxic but fully immunogenic [9-10].

GENETIC DETOXIFICATION OF PT

The failure to induce protective immunity with recombinant subunits suggested that the ideal vaccine should be a PT molecule whose toxicity has been eliminated by genetic manipulation of the gene coding for subunit S1. To do so, we and other investigators generated a number of recombinant S1 molecules containing aminoacid substitutions and tested their enzymatic activity. Substitution of either Arg9, Asp11, Arg13, Trp26, or Glu129 was found to reduce the enzymatic activity to undetectable levels[8]. Each of the above mutations was then introduced into the chromosome of *B. pertussis* whose wild type genes had been deleted. These new *B. pertussis* strains were found to produce molecules indistinguishable from PT in SDS-PAGE which had a toxicity that ranged from 0.1% to 10% of wild type PT. Since even 0.1% of the toxicity is by far too high for a molecule to be used in a vaccine, we combined some of the above mutations and obtained PT double mutants that were at least 10^6 times less toxic than wild type PT (Table II)[9]. Such molecules, being non toxic, were ideal candidates for new vaccines provided they had maintained the correct B- and T-cell epitopes and were able to induce protective immunity in animal models. The non toxic double mutant shown in Table II was found to have the same B- and T-cell epitopes as wild type toxin, to induce toxin-neutralizing antibodies, and to protect mice from the intracerebral challenge[9, 10].

GENETICALLY DETOXIFIED MOLECULES ARE SAFER AND MORE IMMUNOGENIC THAN CHEMICALLY DETOXIFIED TOXINS

Formaldehyde and glutaraldehyde are usually used to detoxify toxins for vaccine purposes. To find whether PT-9K/129G is more immunogenic than chemically inactivated per-

TABLE II

In vivo and *in vitro* properties of PT-9K/129G compared with purified native PT

Property		PT	PT-9K/129G
CHO cell clustered growth	(ng/ml)	0.005	> 5,000
ADP ribosylation	(ug)	0.001	>20
Mitogenicity	(ug/ml)	0.1-0.3	0.1-0.3
Hemagglutination	(ug/well)	0.1	0.1
Affinity constant (anti-S1)	[Ka (L/mol)]	2.4×10^8	6.1×10^8
Affinity constant (anti- PT)	[Ka (L/mol)]	2.0×10^{10}	9.8×10^9
Histamine-sensitization	(ug/mouse)	0.1-0.5	> 50
Leukocytosis stimulation	(ug/mouse)	0.02	> 50
Anaphylaxis potentiation	(ug/mouse)	0.04	> 7.5
Enhance isulin secretion	(ug/mouse)	<1	> 25
In vivo acute toxicity	(ug/Kg)	N.D.	> 1,500

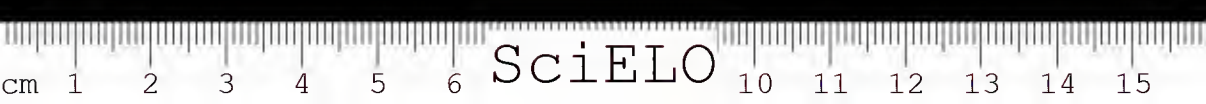
N.D. = not determined

tussis toxin molecules, we treated PT-9K/129G with 0.07% and 0.42% formaldehyde for 48 hours at 37°C and then compared the *in vitro* properties and the immunogenicity or the resulting molecules. As shown in Table III, formaldehyde treatment abolished the hemagglutinating property of PT-9K/129G, decreased its affinity for anti-PT gama globulins and masked the epitope recognized by the protective monoclonal antibody 1B7. The immunogenicity of PT-9K/129G did not decrease after formaldehyde treatment. The ELISA titer of the sera obtained from guinea pigs immunized with natural or formalin-treated molecules were almost identical (Table III). In marked contrast, the ability of the sera to neutralize PT in the CHO assay were dramatically lower when chemically treated molecules were used for immunization. In the intracerebral challenge assay, the formalin-treated molecules were remarkably less potent than natural PT-9K/129G (Table II). We have therefore shown that chemical treatment of toxins for vaccine production induces profound changes in the antigenic properties of the molecules. These changes do not alter the total amount of antibodies induced but change dramatically the quality of the antibodies obtained. As a result, large quantities of chemically treated molecules are required to induce a protective response. Under these conditions, the immune system produces mainly antibodies against non protective epitopes (or with lower affinity for the protective epitopes) and the cellular immunity is overstimulated. Both conditions may favor the appearance of untoward reactions of the Arthus type or delayed typed hypersensitivity. In conclusion, the properties of PT-9K/129G show that molecular genetics has provided new and more efficient tools to inactivate toxins for vaccine use. These molecules have the same conformation as the native proteins and are much better than chemically treated toxins in inducing protective immunity.

TABLE III

Effect of formaldehyde treatment on the properties of the genetically inactivated pertussis toxin mutant PT-9K/129G.

Formaldehyde (%)	Hemagglutination (µg/well)	Affinity Constant: Polyclonal gamma globulins	Affinity Constant: Monoclonal 1B7	Immunogenicity (guinea pigs immunized with 3 µg of antigen): ELISA titer	Immunogenicity: CHO titer	Vaccine Potency (Mice survival after immunization with 5 µg of antigen): Intracerebral challenge
0.0	0.5	$1.15\ 10^{9}$	$5.5\ 10^{7}$	3.5	1/2560	13/16
0.07	4.0	$5.26\ 10^{8}$	-	3.1	1/160	8/16
0.42	>10.0	$6.75\ 10^{7}$	-	3.3	1/80	1/16

CLINICAL STUDIES

After extensive studies in animal models which have shown that PT-9K/129G is non toxic, immunogenic and is able to protect mice from the infection with virulent *B. pertussis*, we have tested PT-9K/129G in human adult volunteers[11]. The results of this study showed that the vaccine was safe and induced a great increase in antibody titers against PT both in ELISA and CHO neutralization assays. The titers of PT-specific antibodies were higher than those reported in similar studies using higher doses of chemi-

cally detoxified PT. In particular, the ratio between toxin neutralizing titers and total anti PT ELISA titers was the highest so far reported, suggesting that also in man, immunization with a molecule not chemically modified induces antibodies with higher affinity for the native PT. After the successful phase I study in adult volunteers, the vaccine is now being tested in 3- and 15-month old children. So far the results confirm the excellent properties of the new pertussis vaccine.

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EVALUATION OF ACELLULAR DPT VACCINES IN INFANTS

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ABSTRACT: Two acellular DPT vaccines containing, as pertussis components, the genetically detoxified pertussis toxin mutant PT-9K/129G, either alone or combined with FHA and 69K, were evaluated for safety and immunogenicity in infants 8-14 months old. Both vaccines induced very mild local reactions which were consistent with the presence of alum and the previous administration of two doses of whole-cell DPT vaccine. A marked increase in specific antibodies to each pertussis component and in pertussis toxin neutralizing antibodies was observed after one dose of either acellular vaccines. All vaccinees also acquired an excellent protective immunity against diphtheria and tetanus, as assessed *in vitro* and *in vivo*.

INTRODUCTION

The genetically detoxified pertussis toxin (PT) mutant PT-9K/129G is naturally devoid of the toxic properties of PT and maintains the physicochemical and the immunological properties of the wild type toxin[1,2].

We have developed acellular pertussis vaccines containing the PT-9K/129G mutant alone or together with the filamentous haemagglutinin (FHA)[3] and an outer membrane protein named 69K or pertactin[4]. These proteins are involved in the adhesion of *B. pertussis* to mammalian cells[5] and therefore, if included in a vaccine, are expected to prevent bacterial colonization. Both FHA and 69K are purified from cultures of the recombinant strain *B. pertussis* W28-9K/129G which produces the non toxic PT mutant PT-9K/

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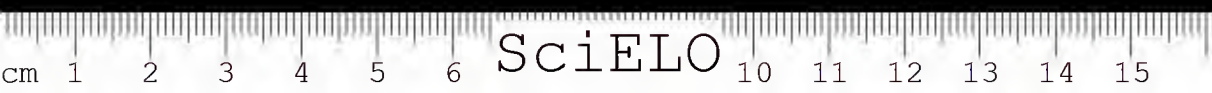
129G and therefore these products cannot be contaminated with activate pertussis toxin. Since no chemical treatment is necessary to inactivate the toxin, these molecules can be safely used in pertussis vaccines without fear of potential reversion to toxicity, loss of immunogenicity, and batch-to-batch variations[6,7].

In previous studies we have shown that acellular pertussis vaccines containing PT-9K/129G alone[8] or combined with FHA and 69K[9] are extremely safe in adult volunteers and that are able to induce high levels of humoral and cellular immunity. These results are confirmed by phase II clinical trials in infants and children which are now in progress. Since it is likely that one of these pertussis formulations will be used for mass immunization and administered within the DPT vaccination schedule, we have prepared two trivalent vaccines, in which diphtheria and tetanus toxoids have been combined with only PT-9K/129G (DPT3/P/AH) or with PT-9K/129G, FHA and 69K (DPT7/PFK/AH).

In this paper we report the safety and the immunogenicity in infants of these new acellular DPT vaccines.

MATERIALS AND METHODS

Study design. Forty-five healthy infants of both sexes, 8 to 14 months of age, were recruited at the Health Local Unit of Ozieri, Sardinia, Italy. Among these, twenty-one and twenty-four subjects received intramuscularly one dose of DPT3/P/AH or DPT7/PFK/AH vaccine, respectively. All infants had previously received at least two doses of conventional whole-cell DPT vaccine.

Safety assessment and serology. Rectal temperature was monitored at 3, 6 and 24 hours after vaccination. Drowsiness, fussiness, appetite, vomiting, redness, swelling and pain were monitored 3 and 6 hr after vaccination and then at bed-time throughout the first week and on the 14^{th} evening.

For redness and swelling we have reported in table 1 only values which were ≥ 1 cm; for fussiness and appetite only values monitored throughout the first three days after vaccination.

Parents were instructed to record the rectal temperature of the infant and to evaluate any local and systemic reactions. All infants were home-visited 24 hs after the vaccine ad-

TABLE I

Adverse reactions after vaccination

Reaction	DPT3/P/AH	DPT7/PFK/AH
Fever	2/21	3/24
Drowsiness	2/21	3/24
Fussiness	5/21	6/24
Appetite	4/21	4/24
Vomiting	2/21	2/24
Redness	4/21	1/24
Swelling	4/21	3/24
Pain	2/21	6/24

ministration by a follow-up nurse who monitored parents by phone throughout 14 days.

Venous blood samples for *in vivo* and *in vitro* evaluation of specific IgG and toxin-neutralizing antibodies were obtained before and 4 weeks after vaccination from 7 and 11 infants receiving, respectively, the DPT3/P/AH or the DPT7/PFK/AH vaccines.

Vaccines. Both the acellular DPT vaccines were prepared at Sclavo Laboratories (Siena, Italy). Each 0.5 ml single dose vial of DPT3/P/AH contained 15 Lf of diphtheria toxoid, 10 Lf of tetanus toxoid, 10 μg of PT-9K/129G, 0.05 mg of thimerosal and 2 mg of aluminium hydroxide. Each 0.5 ml single dose vial of DPT7/PFK/AH contained 20 Lf of diphtheria toxoid, 12.5 Lf of tetanus toxoid, 5 μg of PT-9K/129G, 5 μg of FHA, 3 μg of 69K, 0.05 mg of thimerosal, and 1 mg of aluminium hydroxide.

Antigens. The non toxic mutant PT-9K/129G and FHA were purified from the culture supernatants of the recombinant strain *B. pertussis* W28-9K/129G[1] according to a modified version of the method described by Cowell et al.[10]. The 69K protein was extracted from cell paste of *B. pertussis* W28-9K/129G[1] by heat treatment and purified to the homogeneity by anionic exchange chromatography and gel filtration (Manetti & Rappuoli, manuscript in preparation). Before use, each antigen underwent a mild stabilization with formaldehyde.

Diphtheria and tetanus toxoids were obtained as previously described[11].

CHO cell toxin neutralization assay. Pertussis toxin-neutralizing antibodies induced by vaccination were using, as standard, the U.S. Reference Human Pertussis Antiserum (lot # 3), containing 640 neutralizing units, kindly provided by the Center for Drugs and Biologics, Bethesda, MD. U.S.A. Briefly, sera from volunteers obtained after administration of one or two doses of PFK/2 vaccine were diluted directly in the wells of flat-bottomed microplates (Costar, Cambridge, MA, U.S.A.) in 25 μl of Dulbecco's Modified Eagle Medium (DMEM, Flow Laboratories, Mc Lean, VA, U.S.A.). Purified wild type PT (120 pg) in 25 μl of Coulter medium DMEM was added to each well and the plates were incubated for 3 h at 37°C. After the incubation period, 0.2 ml of DMEM containing $1x10^4$ CHO cells, previously treated with 1 mg/ml of trypsin, were added to each well and incubated for 48 h at 37°C in atmosphere of 5% CO_2. As positive control, the clustering effect of PT alone was titered in each plate. Neutralizing titers were expressed as the reciprocal of the highest serum dilution causing complete inhibition of the clustering activity induced by the native toxin.

Vero cell toxin neutralization assay. Diphtheria toxin-neutralizing antibodies induced by vaccination were tested by the VERO cell assay using as standard the NIH Reference Antiserum (lot # A50), containing 3,200 neutralizing units. The test was performed in the wells of flat-bottomed microplates (Costar, Cambridge, MA, U.S.A.). Antisera from vaccinated infants and the NIH reference serum were diluted in culture medium M199, containing 10% FCS, 2 mM glutamine, 25 mM Hepes, 50 μg/ml gentamicin, directly in the wells by twofold serial dilutions. The volume of antisera added to each well is 20 μl. After that, the diphtheria toxin (80 LF/ml diluted $1:10^5$) was added to each well in a volume of 20 μl/ml. The plates are then incubated for 3 h at 37°C. After the incubation time, 200 μl of culture medium containing 10^9 VERO cells are added to each well. The microtiter plates were then incubated at 37°C and, 48-72 h later, cells were strained with crystal violet and the stain was solubilized with 50% solution in water (vol/vol) of ethanol. Absorbance values at 560

nm of samples were measured against blank on a Titertek Multiskan (Flow Laboratories, Inc., McLean, VA, U.S.A.). Controls for each plate included wells with cells and diphtheria toxin (positive control), and wells with cells but no toxin (negative control). Each serum sample was tested in duplicate and absorbance values were averaged. The neutralizing titer is expressed as the reciprocal of the highest dilution of serum samples giving absorbance values comparable to those of the negative control (100% of protection).

Elisa

The ELISA method was performed as previously described[2,8]. Wells of flat-bottomed polystyrene microtest plates (Dynatech Laboratories, Inc., Alexandria, VA, U.S.A.) were coated with 100μl of PBS pH 7.4 containing 1 μg of purified PT or FHA or 69K, diphtheria toxoid or tetanus toxoid. The coating was performed for 2 h at 37°C and overnight at 4°C in a humidified chamber. The coating buffer was aspirated, and wells were washed with 200 μl of PBS containing 0.05% Tween 20 and 0.02% sodium azide (PTA). To minimize non-specific adsorption of serum proteins to the plastic, wells were coated with 200 μl of a blocking solution consisting of 1% bovine serum albumin (BSA) in PBS, and then incubated for 2 h at 37°C. Plates were then washed three times in PTA and 200 μl of fivefold diluted test serum were added to the wells. The U.S. Reference Human Pertussis Antiserum (lot # 3) containing 200 ELISA Units (EU) per ml of IgG anti-PT and 200 EU/ml anti-FHA was used as reference standard. In the case of the 69K protein, we used an "in-house standard" immune serum to which we assigned a value of 20 EU/ml of IgG anti-69K, as well as 10 EU/ml of IgG anti-diphtheria and anti-tetanus toxoids. Following incubation at 37°C for 2 h, plates were washed three times with PTA and a conjugate of anti human IgG-alkaline phosphatase was added. Plates were then incubated at 37°C for 2 h and washed three times with PTA. Finally, 100μl of p-nitrophenyl phosphate substrate (Sigma Chemical Co., St. Louis, MO, U.S.A.) 1mg/ml in 1 M diethanolamine, pH 9.8, containing 1 mM $MgCl_2$, was added to each well. The enzyme-substrate reaction, which developed at room temperature, was stopped after 30 min, and the absorbance values of the samples was measured at 405 nm against blank (substrate in diethanolamine, pH 9.8) on a Titertek Multiskan (Flow Laboratories, Inc., McLean, VA, U.S.A.). Controls for each plate included wells with serum samples but no antigen, and wells with antigen but no serum samples. Each serum sample was tested in duplicate and absorbance values were averaged. Total IgG antibodies to PT, FHA and 69K in the test samples were expressed as geometric mean of EU/ml, determined according to the parallel line bioassay procedure described by Manclark et al.[14].

Diphtheria and tetanus antitoxin tritation in vivo. The assays were performed according to W.H.O. recommendations using rabbits for diphtheria and mice for tetanus antitoxin tritation. The results, expressed in International Units (IU) per ml, were obtained according to the N.I.H. reference diphtheria antiserum (Lot # A50, containing 6 IU/ml) and the N.I.H. reference tetanus antiserum (Lot # A50, containing 6 IU/ml), used as standards.

RESULTS

Safety. The follow-up performed after the administration of DPT3/P/AH (table 1) showed that no infants had fever over 38°C with two exceptions (38.1°C and 38.2°C). However, in both cases, the temperature was over 38°C for only one measurement

and, in no cases, the administration of anti-pyretic drugs was necessary. Unusual drowsiness was only noticed in two infants. Five out of twenty-one infants were more irritable than usual throughout 72 hr following vaccination, but all of them maintained a normal activity. Three infants had loss of appetite (two of them after the first three days), while two infants had an isolate episode of vomiting on day 2 and 4, respectively. Four out of twenty-one subjects had redness and swelling at the site of injection and finally, two infants had local pain that in one case was very mild.

As far as vaccination with DPT7/PFK/AH is concerned, the follow-up of adverse reactions (table 1) showed that only one out of twenty-four infants had redness and three had swelling. Local pain was reported in six infants. Three infants had fever over 38°C. Drowsiness in the first twenty-four hours after vaccination was noticed in three infants. Mild fussiness occurred in six subjects and for four infants, parents reported loss of appetite throughout 72 hr after vaccination while two infants had isolate episodes of vomiting.

Serology. The humoral response of infants to the DPT3/P/AH vaccine is reported in figure 1 and table 2 and that one to DPT7/PFK/AH vaccine is reported in figure 2 and table 2.

As expected, all the infants, after two doses of conventional DPT cellular vaccine, showed low titers of anti-PT, anti-FHA and anti-69K IgG antibodies, assayed by ELISA (Figures 1, 2), but a good immune response against diphtheria and tetanus, either evaluated as passive protection *in vivo* (Table 2) or as specific antibodies *in vitro* (Figures 1, 2).
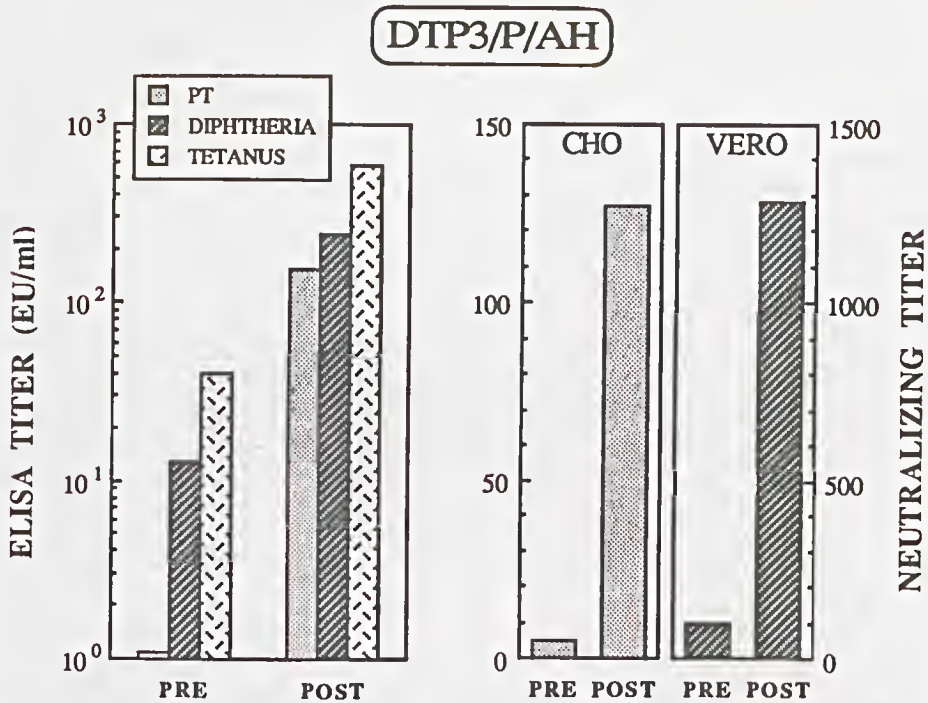
Figure 1 — Specific IgG antibodies (ELISA) as well as pertussis (CHO) and diphtheria (VERO) toxin neutralizing antibodies in infants receiving DPT3/P/AH vaccine.
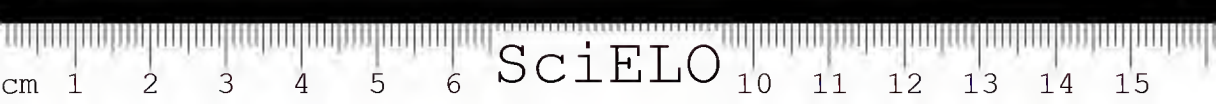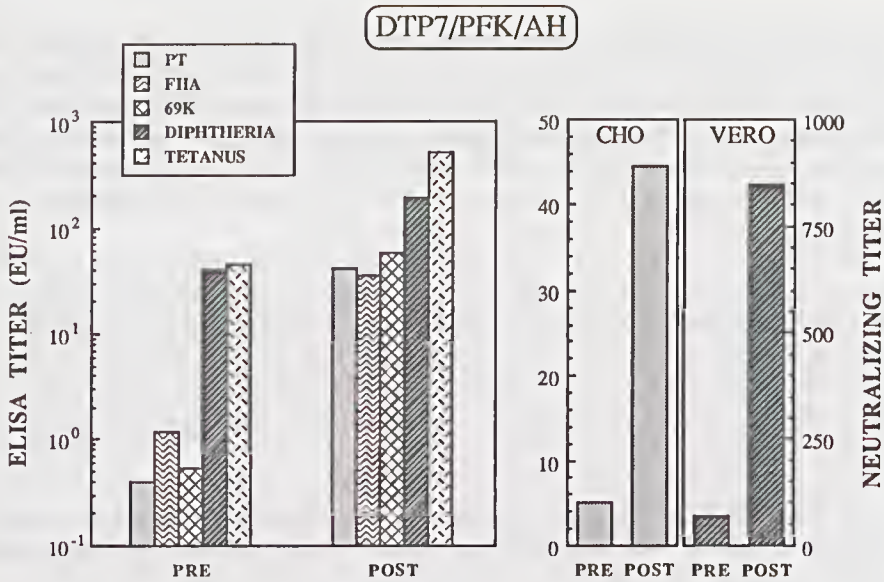
Figure 2 — Specific IgG antibodies (ELISA) as well as pertussis (CHO) and diphtheria (VERO) toxin neutralizing antibodies in infants receiving DPT7/PFK/AH vaccine.

TABLE II

Diphtheria and tetanus antitoxin titration *in vivo*[1]

Vacine	Infant	Diphtheria		Tetanus	
	Nº	PRE	POST	PRE	POST
DPT3/P/AH	10	< 0.01	> 0.1 < 1	1	> 8 < 15
	11	1	> 2 < 4	2	> 8 < 15
	12	> 0.1 < 1	4	1	15
	15	> 0.1 < 1	> 4	1	> 15
	16	N.D.[2]	N.D.[2]	N.D.[2]	N.D.[2]
	17	> 0.1 < 1	4	> 1 < 2	> 8 < 15
	19	> 0.1 < 1	> 4	4	> 15
DPT7/PFK/AH	1	> 1 < 2	> 4	> 2	> 15
	2	> 0.1 < 1	> 2 < 4	> 1 < 2	> 15
	3	> 0.1 < 1	> 2 < 4	> 1 < 2	> 15
	6	0.1	> 1 < 2	> 1 < 2	> 15
	7	> 0.1 < 1	> 1 < 2	> 1 < 2	> 8 < 15
	8	> 0.1 < 1	4	> 2 < 4	> 15
	10	N.D.[2]	N.D.[2]	N.D.[2]	N.D.[2]
	12	> 0.1 < 1	> 4	> 1 < 2	> 15
	17	> 1 < 2	4	> 2 < 4	> 8 < 15
	18	> 0.1 < 1	> 1 < 2	> 2 < 4	> 15
	19	0.1	1	> 1 < 2	> 8 < 15

1) The test was performed in rabbits and in mice for *in vivo* titration of anti-diphitheria and anti-tetanus neutralizing antibodies, respectively. Results are expressed as International Units (IU)/ml.
2) N.D. Not Determined.

Total IgG antibodies to each vaccine components increased after the administration of the acellular DPT vaccines. A similar rise was noted for pertussis toxin-neutralizing antibodies, evaluated by the CHO cell assay, and for diphtheria toxin-neutralizing antibodies, assayed by the VERO cell assay (Figure 1, 2). A high serum activity against diphtheria and tetanus toxins was also observed in animals, using the *in vivo* assay recommended by WHO. In fact, sera from infants receiving one dose of either DPT acellular vaccine showed a marked increase of passive protection against diphtheria and even more pronounced protection against tetanus. In most cases the *in vivo* diphtheria antitoxin titers were over 4 IU/ml and the tetanus antitoxin titers were over 15 IU/ml (Table 2).

DISCUSSION

In a previous phase I[8,9] and a phase II (Podda et al., manuscript in preparation) clinical study, we had carefully tested the safety and the immunogenicity of two monovalent acellular pertussis vaccines, one containing the genetically detoxified pertussis toxin mutant PT-9K/129G[1] and the other containing PT-9K/129G combined with FHA and 69K. Both vaccines proved to be safe and immunogenic in adults and children. Since the final formulation of pertussis vaccine, to be introduced in the vaccination schedule of children, is expected to contain also diphtheria and tetanus toxoids, we have prepared two acellular DPT vaccines (DPT3/P/AH and DPT7/PFK/AH) which pass the test of the American and European pharmacopea and we have tested them in infants.

Although the present study is a not controlled open evaluation carried out on a limited number of subjects, some comments about the safety and the immunogenicity of these acellular DPT vaccines can be made.

The local reactions reported after vaccination with DPT3/P/AH were very mild and consistent with the administration of a vaccine adsorbed with aluminium hydroxide (2 mg/dose) and with the fact that all infants had, previously, received two doses of conventional whole-cell DPT vaccine. In detail, the 19% of infants had redness of 1 cm of diameter at the site of injection, but in only one case the size of the reaction was greater than 1 cm. Swelling and local pain occurred in the 19% and 9% of infants, respectively. The most frequent systemic reaction was an unusual irritability, reported in the 23% of subjects, which, however, did not affect the normal activity of infants. In two cases (9.5%), moderate fever and excessive sleeping were reported.

As compared with DPT3/P/AH, the local reactions, mainly redness, were less frequent after the administration of DPT7/PFK/AH vaccine. This is probably due to the reduced amount of adjuvant (1 mg versus 2 mg). In detail, only one infant had redness at the site of injection while swelling and local pain occurred in the 12.5% and 25% of infants, respectively.

Although the major aim of this small trial was the evaluation of the safety we have also performed an immunogenicity study in a small number of infants from whom it was possible to obtain serum samples before and one month after vaccination with the acellular DPT vaccines. All infants had a low level of anti-PT, anti-FHA and anti-69K antibodies assessed by ELISA and a not detectable PT-neutralizing activity. Both specific and neutralizing antibodies markedly increased after the administration of either vaccines but the enhancement of the humoral immunity, probably due to the higher amount of adjuvant, was more pronounced after vaccination with DPT3/P/AH. Also the

serum antibody responses to diphtheria and tetanus toxoids, which was already good before vaccination, augmented more than ten times after one dose of either acellular DPT vaccines as evaluated *in vitro* in terms of ELISA and VERO titers. The enhanced serum activity against diphtheria and tetanus was confirmed by *in vivo* antitoxin antibody titrations. In fact, sera from all vaccinees were able to confer a high passive protective immunity to the animals reaching in most cases, after one dose of acellular DPT vaccine, values of more than 4 IU/ml against diphtheria and even more than 15 IU/ml against tetanus.

In conclusion, the careful follow-up of 21 infants receiving DPT3/P/AH and of 24 infants receiving DPT7/PFK/AH in addition to the immune response evaluation performed in some of them proved the substantial safety and the excellent immunogenicity of these acellular DPT vaccines and encourages their further evaluation in enlarged clinical trials.

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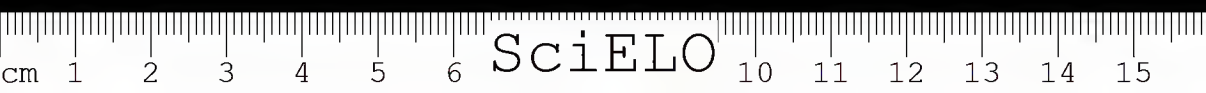
CONTRIBUTION OF MOLECULAR BIOLOGY TO VACCINE DEVELOPMENT AND MOLECULAR EPIDEMIOLOGY OF RABIES DISEASE*

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I. RABIES DISEASE: AN HISTORICAL EXAMPLE OF INTERNATIONAL COOPERATION, NOTABLY BETWEEN BRAZIL AND FRANCE

In order to build an establishment devoted to the treatment of rabies disease based on Louis Pasteur's famous "method of prevention of rabies disease after biting" [45], a worldwide foundation was organized. Besides numerous anonymous contributors, the four first donators were the Tsar of Russia, the Sultan of Turkey, Madame Boussicot and the Emperor of Brazil, Dom Pedro II who crystalized by this action, a constant friendship with Louis Pasteur. This first authentic example of international financial and scientific collaboration resulted in the founding of the Pasteur Institute of Paris inaugurated in 1888, and inspired numerous other projects throughout the world to fight the rabies disease raging at the end of the 19th century. As early as 1888, Dom Pedro II organized the building of a Pasteur Institute in Rio de Janeiro directed by Dr Ferreira dos Santos who had studied the method of rabies prophylaxis in Paris [46]. Today, the brazilian production of anti-rabies vaccines is dispersed in four places including the Butantan Institute of São Paulo, which celebrates its 90th Anniversary in 1991.

II. CAUSES AND CONSEOUENCES OF RABIES DISEASE WORLDWIDE TODAY

Human deaths by rabies disease are estimated as at least 50,000 per year, although the WHO records less alarmist statistics due to the difficulty of collecting data from some

* *Summary of two conferences during the symposium.*
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countries (World survey of rabies 1988 no 24, WHO/Rabies/91.202). China and India are probably the most contaminated (25,000 cases each) followed by the Philippines, Thailand and Indonesia in Asia, most of Africa, Mexico and Central America. In South America, besides human cases (200 to 500 in Brasil) rabies disease is also an economical problem, responsible for considerable losses of cattle, approximately 500,000 per year[1]. Indeed, the only continent to remain totally rabies-free is Oceania together with several island countries such as Japan, United Kingdom, Ireland, as well as certain Antillas.

The perenniality of the disease is ensured by several wild animal species serving as natural virus reservoirs. These reservoir species, also acting as vectors, are characterized by their high susceptibility to the virus and their propensity to transmit the infection before dying. They must be distinguished from the other species (cattle, human, etc...) that constitute a "cul de sac" for the infection.

The vector species are susceptible to considerable variation depending both on geographic and temporal criteria[10, 58]. Today, for example, the dog is the main reservoir in Asia, Africa and Latin-America, the raccoon and the skunk in North-America, the vampire bat in Central- and South-America, the red fox in Europe. Fox rabies is relatively recent in Western-Europe where it penetrated from the far East (USSR). It first appeared in France in 1968, replacing the dog or "street" rabies contemporary of Louis Pasteur which disappeared in 1920-1930[60]. Another recent european vector is the insectivorous bat, initially described in the North of Europe and invading progressively towards the South. The first french case was diagnosed late 1989[61], others being reported in Spain. Bat rabies is today a major problem because of its divergence from the "classical" rabies viruses (see § VI.1).

As reflected by the great variability of the host, rabies disease is caused by a group of different agents describing the *Lyssavirus* genus of the *Rhabdoviridae* family (figure 1)[4].

RHABDOVIRIDAE FAMILY

LYSSAVIRUS GENUS

serotype	geographic distrib.	animal species
1. Rabies	*world* except Oceania, U.K., Japan, islands...	carnivores cattle, bats human
2. Lagos bat	*Africa:* Nigeria, Zimbabwe Cent. Afri. Rep., South-Africa, Senegal	frugivorous bats cats
3. Mokola	*Africa:* Nigeria, Zimbabwe Cent. Afri. Rep., Cameroon	shrews, rodents cats, dogs, human
4. Duvenhage	*Africa:* Zimbabwe South-Africa	insectivorous bats human
unclassified		
Obodhiang	*Africa:* Sudan	Mansonia
Kotonkan	*Africa:* Nigeria	Culicoids
EBL (European Bat Lyssavirus)	*Europe:* Finland, France Poland...	insectivorous bats human

Figure 1 — Classification of the *Lyssavirus* genus.

These are classified on the basis of serological and antigenic relationships into four serotypes[13, 32]. Serotype 1 comprises the vaccinal strains and the "classical" wild rabies viruses. Serotype 2, 3 and 4 are the so called "rabies-related" viruses because of their distant relationship with serotype 1; serotype 3 corresponds to Mokola virus; serotypes 2 and 4 group are bat viruses, represented by Lagos bat and Duvenhage isolates, respectively. The recent european bat isolates form the European Bat Lyssavirus (EBL) group, as yet unclassified[61].

III. BRIEF DESCRIPTION OF THE DISEASE

Rabies is a viral infection of the nervous system affecting particularly mammals where it causes an acute encephalitis[4]. The virus generally penetrates by effraction (essentially biting) although some unclassical routes of infection by aerosols or licking of mucous membranes have been described[19]. At the site of the bite, generally in the external tissue, a local multiplication is believed to occur but not obligatory[56]. The virus is clearly neurotropic and tends to infect neurons of the peripheral nervous system[71]. The putative receptor of the rabies virus is as yet unknown. On the basis of sequence homology between the external glycoprotein of the virus and the receptor binding site of venom snake neorotoxins, it was postulated that the nicotinic acetylcholine receptor could be the rabies receptor [7, 39]. But if this is possible in muscular cells, it seems that at the level of fibroblastic and above all neuronal cells, the rabies receptor(s) is(are) more complex, also involving oligosaccharides or lipoproteinic elements such as the sialic acids of gangliosides[71, 78].

Once in the nerve, the virus replicates and ascends to the central nervous system by retrograde axoplasmic flow. Some regions of the central nervous system are preferentially infected such as the cortex, the pons or the thalamus[71]. Late in infection, all the central nervous system is infected as well as certain external tissues, such as the salivary glands, that allow the perenity of infection. Rabies disease is characterized by a variable incubation period (generally 1-3 months), contrasting with a short and violent symptomatic period (less than 1 week) leading invariably to death in the absence of any possible therapy[4]. Generally no evident histopathological perturbation accompanies death, as if the virus killed the organism without killing the cell. Several electrophysiological dysfunctions, notably at the level of the paradoxical sleep, have been noted in infected mice[29].

IV. STRUCTURAL AND FUNCTIONAL STUDY OF THE VIRION (FIGURE 2)

1. Morphology, structure

If Pasteur was already certain that rabies disease was caused by a virus, over 80 years of technical progress was necessary, in tissue culture and electron microscopic domains, before observing the first rabies particule in 1963, notably at the Pasteur Institute[3, 21]. The virion has a bullet-shaped form with a round extremity and a flat base. The unique single-stranded RNA genome and the five viral-encoded proteins are distributed in two structural and functional units[67]: a central dense helical cylinder corresponding to the ribonucleocapsid surrounded by a lipoproteinic envelope obtained from

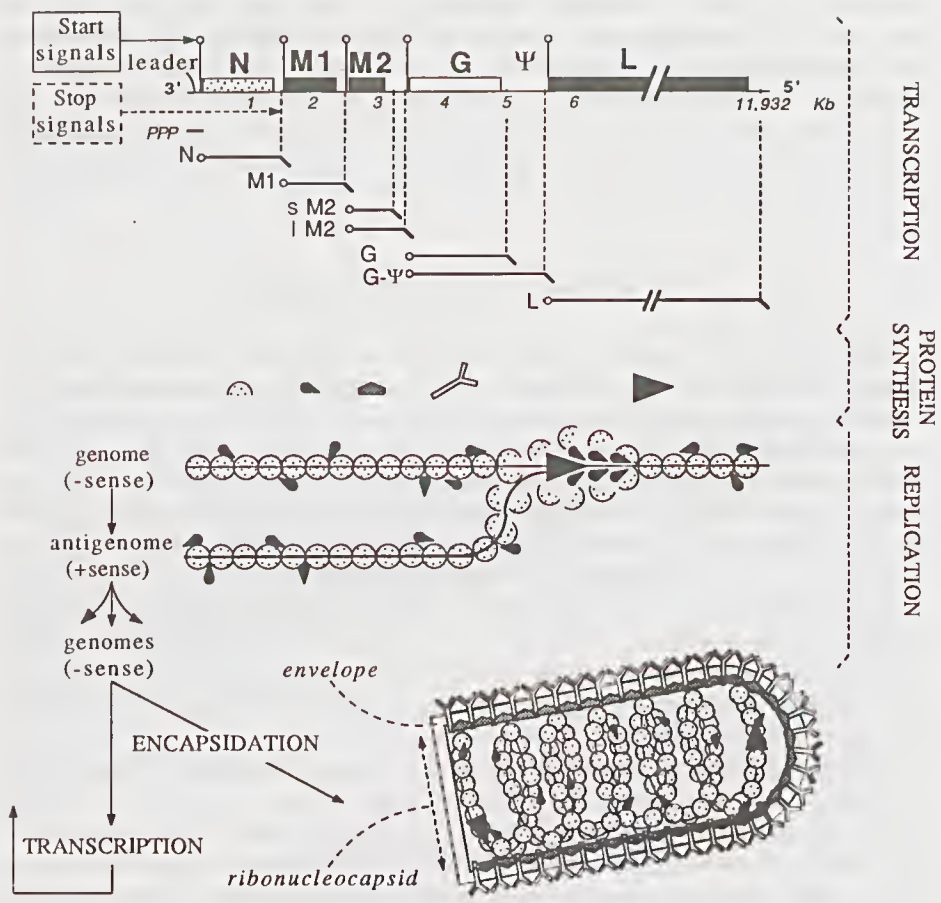



Figure 2 — Structure, transcription and replication mechanisms of rabies genome.

the host-cell surface during the budding of the virion. The ribonucleocapsid is constituted by the RNA genome tightly associated with the N nucleoprotein, and less stringently with the M1 phosphoprotein and the L polymerase. The RNA-N protein association is so intimate that the genome is completely insensitive to RNase. The envelope carries the M2 membrane or matrix protein on the inner side and is traversed by the G glycoprotein which forms spike-like glycosylated projections towards the outside. As is evident from their names, the G glycoprotein is glycosylated and the M1 phosphoprotein is phosphorylated. The N nucleoprotein is also phosphorylated.

The rabies genome is an unsegmented RNA molecule of negative polarity. Negative means that it is not directly translatable by the cell machinery, but forced to assure an autonomous transcription step by producing positive stranded mRNAs, as soon as it penetrates the cytoplasm. Unsegmented means that cistrons are juxtaposed, limited by start and stop transcription signals and separated, by intergenic regions. This genomic organization is shared by the *Rhabdoviridae* and *Paramyxoviridae* families which have consistently envolved an identical multiplication strategy.

2. Multiplication Strategy

Once fixed on its putative receptor, the virus penetrates the cell by pinocytosis and fuses its envelope with that of the lysosomal vacuole. The ribonucleocapsid is thereby liberated into the cytoplasm to serve as a template for transcription and replication mechanisms in this genuine form, without any decapsidation. These mechanisms have been largely inspired from the vesicular stomatitis virus (VSV) model, far the more extensively studied unsegmented negative stranded RNA virus[5, 26, 73, 74].

The transcription occurs from the 3' to the 5' end of the genome, producing monocistronic transcripts. First a small noncapped, nonpolyadenylated leader RNA, then five capped and polyadenylated messenger RNAs (mRNAs) successively corresponding to the N, M1, M2, G and L proteins. This transcription is sequential and of decreasing efficiency, meaning that a messenger is always transcribed after the 3' proximal one and at lower rate. Consequently, the extent of gene expression is directly related to the genomic location. It appears that the transcription complex stops at the end of each cistron, and reinitiates only partially at the beginning of the following one. This sequential progression is dictated by the ten nucleotides long start and stop transcription signals recognized by the running transcription complex.

It is only after the translation of the mRNAs into the corresponding proteins that the switch to replication step occurs. This suggests that at least one of the viral protein is involved in this switch. The N nucleoprotein ratio is currently thought to be the key point, the replication beginning only if sufficiently large amounts of N protein are available for encapsidation. The replication leads to the synthesis of a full length positive stand antigenome that, in turn, will serve as a template to amplify the negative strand genomes. These will be either encapsidated in the progeny virions or submitted to a secondary transcription step.

Although not yet studied as completely, the rabies genome expression is coherent with the VSV model but shows typical features:

1) the presence of very variable intergenes both in size and nucleotidic composition, notably between G and L cistrons (423 bases)[67, 69];

2) the presence of two consecutive stop signals for the G and M2 cistrons, alternatively used to produce either a large or a small messenger. Because the transcription complex is thereby released more or less far upstream from the distal start signal, alternative termination influences the extent of distal gene transcription. It is a typical regulatory mechanism since the ratio between both messengers varies during the course of infection, and differently in fibroblastic or neuronal cells[64, 65, 66].

3. Functional Role of the Viral Proteins

The ribonucleocapsid structure (N protein coated RNA genome, M1 and L proteins) is a functional entity autonomous in transcription and replication. Typically, the N encapsidated genome is recognized as a template by the viral polymerase composed of two functional elements. While the L protein is the actual RNA-dependent RNA polymerase carrying most of the required activities (RNA synthesis, capping and polyadenylation), the M1 seems more devoted to regulatory functions. It ensures notably a local decapsidation of the template upstream from the running L protein, by displacing the N proteins to leave the template accessible for the polymerase. The affinity of the N protein molecules for the M1 phosphoprotein results from the extreme electronegativity of the latter

that mimics the RNA backbone. This electronegativity is due both to the richness of the M1 protein in acidic amino-acids and to the presence of the phosphate residues. Downstream the polymerase complex, the N nucleoproteins immediately re-encapsidate the genome, suggesting an exact coordination between polymerization and encapsidation.

The M2 membrane protein, occupying an intermediate position between the envelope and the ribonucleocapsid, interacts with both units. Thereby, it plays a capital role during the maturation step which precedes the budding of the virion out of the cell. At the ribonucleocapsid level, it inhibits the transcription and replication mechanisms and catalyses a strong condensation in an helical structure[40]. At the membrane level, it designates the places where the virus will bud by facilitating local concentration of the G glycoprotein on the cell surface. The external spike-like glycosylated projections of the G protein constitute the major viral antigen and are likely to mediate the binding to the target cellular receptor although the binding site remains uncharacterized.

V. RABIES VACCINES: A CENTENARY OF CONTINUAL TECHNICAL PROGRESSES FROM PASTEUR'S "DESSICATED SPINAL CHORDS" TO GENETIC ENGINEERING

1. Classical Vaccines

To fight the disease, the first serious scientific approach was undertaken by Pierre-Victor Galtier and subsequently Louis Pasteur, to find an effective vaccine. Since the famous and successful Pasteur's injections of dessicated spinal chord of rabid rabbits[45], considerable efforts were undertaken to increase the efficiency and safety of vaccines. They were subsequently prepared from infected brains of adult animals, then of suckling mice to avoid risks of encephalopathy, and finally from infected cell cultures[17, 34, 59]. In parallel, performing methods to purify (zonal centrifugation) and inactivate (UV, B-propriolactone) the virus were developed.

2. Subunit Vaccines without DNA Technology

The project of producing rabies subunit vaccines consists of using purified viral polypeptides or part of polypeptides for immunization. Biochemical techniques to purify viral polypeptides and to restructure them in a convenient form were first assayed. The purified glycoprotein was anchored either on an oligosaccharide bone composed of glycoside Quil A, or on a lipidic membrane, giving rise to "rabies iscoms" and "rabies immunosomes", respectively[48]. Even though both products showed a high protective activity in pre- or post-exposure tests, the difficulties inherent to glycoprotein purification rendered impossible the development of a vaccine for commercial reasons. DNA-recombinant technology could bypass this difficulty by producing substantial quantities of viral polypeptides. Alternatively, a vaccinal approach by synthetic peptides corresponding to B- or T-cell epitopes coupled to "carrier" molecules was investigated[17].

3. Contribution of the DNA-recombinant Technology to Rabies Vaccine

The different steps for the general strategy to produce a rabies vaccine by DNA-recombinant technology are (figure 3): 1) The choice of the viral antigen eliciting the

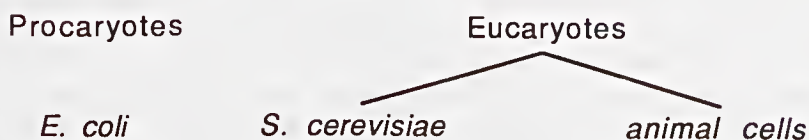
best immune response; 2) The choice to work either with the corresponding messenger RNA or the total RNA genome; 3) After the reverse transcription into complementary DNA (cDNA) and the cloning, the choice of the expression system, including the vector, the promotor and the host-cell system.

GENERAL STRATEGY FOR A DNA-RECOMBINANT VACCINE

1) choice of the viral antigen

2) reverse transcription of the RNA gene
of the messenger RNA

3) cloning of the complementary DNA

4) choice of the expression system
(host cells - vector - promotor)

5) purification of the recombinant protein
(unecessar when using virus vectors able to multiply on the targeted animal)

6) protective power on animal

Figure 3 — Strategy towards a genetic engineered vaccine.

Assuming that expression is good, then the recombinant protein has to be purified, except when using virus vectors able to multiply in the target animal itself, such as vaccinia vectors. Finally, the last and crutial step is to test the protective power of the recombinant on animals.

A. Choice of the Viral Antigen

Although all viral proteins show antigenicity, they do not all play the same role in protection[17]: the purified G protein has a protective effect against an intracerebral challenge with rabies virus while the purified ribonucleocapsid is only protective against a less stringent peripheral challenge (intramuscular)[25]. Consistently, the glycoprotein is the only viral protein having the capacity to elicit virus neutralising antibodies[75]. This property is mainly dependent on the preservation of its three dimensional structure, although a linear neutralising epitope was recently characterized[16]. On the other hand, it shares the capacity to induce a cellular immune response involving both helper and cytotoxic T lymphocytes with other proteins of the ribonucleocapsid, namely the N and the M1 proteins[17]. The major epitopes for both B and T cells have been located along the viral proteins[24].

These studies suggest that if the glycoprotein is obviously the most important antigen for vaccination, the nucleoprotein could represent an interesting enhancer for two principal reasons: 1) because of its capacity to substantially increase the helper T cell immune response as is reflected by the numerous T-cell epitopes along the N protein; 2) because it is a less variable antigen,[13, 67, 68] and could increase the spectrum of a vaccine, notably to rabies-related viruses.

B. Working with Messenger RNAs or Total Genome

An mRNA template is easy to prime with an oligo (U), but impairs the study of the untranscribed intergenic regions separating the cistrons, which could be important in regulating transcription. This study is only accessible when cloning the whole genome but the latter is not polyadenylated at the 3' end. This obliges the need for a specific primer deduced, for example, from the direct chemical sequence of the 3' end of the RNA genome[68]. During the last ten years of rabies molecular biology, both strategies were adopted. The first rabies sequence to be published predicted the glycoprotein structure of the ERA strain, and was obtained by the intermediate of its mRNA[2]. Alternatively, the genome of the PV strain was the first to be cloned to completion by using three consecutive specific primers, and the sequence of its 11932 nucleotides determined[68, 69, 70].

Today, the structure of numerous rabies mRNAs from different strains are known[64, 78]. The genome of the AvO1[49] and SAD B19[20] viruses, mutants of the CVS and SAD strains, respectively, as well as the genome of the rabies-related Mokola virus[15] have been cloned and totally or partially sequenced. All these cloned genes were then available for expression. However, for historical reasons, almost all the expression assays were tempted on the ERA and CVS glycoproteins, the two first genes to be cloned[2, 79]. Now, the tendency is more towards diversity both at the level of the nature (G or N protein) and serotype (strains of rabies or rabies-related viruses) of the expressed antigen.

C. Choice of the Expression System

The key point is then to select an expression system able to perform the required post-translational modifications to make the recombinant polypeptides identical to the authentic protein.

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Antig.	Rabies strain	Host	Vector	Promotor	%	Notes		Ref.
G	ERA	E. Coli	plasmid	lac UV5	2-5%	-inducible promotor -unglycosylated -unmatured (expression without signal peptide) -denatured (reduced) -non protective		42
G	CVS		pBR 322	tryp	2-3%			79
G	ERA		plasmid	M13 phage	25%			36,37
G	ERA		M13 phage	lac (B-Gal fused)	?			38
G	ERA	S. Cere-visiae	2 microns plasmid		weak	-glycosylated		31,37
G	PV	high eucaryote cells -CEF -BHK -VERO -human -etc...	plasmid	SV40 late Herpes TK	weak			64
G	ERA		Bovine papilloma replicon	TK gene	weak			31,37
G	ERA		SV40		?			37
G	ERA		Adenovirus		?			37
G	CVS		Adenovirus	SV40 early Adeno late Adeno E3	protective power in vivo	Injection: high Oral : high		52
G	ERA		Vaccinia (Copenhagen)	7,5 KD early-late		Injection: high Scarific : high Oral : high	side effects	30
G	CVS		Vaccinia (N. Y. Board of Health)	7,5 KD early-late 11 KD late		Injection: high Scarific : high Oral : weak	less side effects	27
G	CVS		Raccoon pox	7,5 KD early-late 11 KD late		Injection: high Oral : high	host specificity	28,41
G	ERA		Fowlpox	H6 early-late		Injection: high	host specificity	62,63
G	CVS	Spodoptora Frugiperda	Baculovirus	polyedrin	good	Protective power by Injection: high		51
G	MOK		Baculovirus	polyedrin	good	Protective power by Injection: high		14
N	CVS	high eucaryote cells	Vaccinia (Copenhagen)	7,5 KD early-late				9
N	CVS		Raccoon pox	7,5 KD early-late 11 KD late	high	Dose dependent protection by injection or tail scratching		41
N	CVS	Spodoptora Frugiperda	Baculovirus	polyedrin	high	Products for diagnosis reagents		8,50,53

Figure 4 — Summary of the systems employed to express the G and N genes of Lyssaviruses.

The glycoprotein is a classic transmembrane protein with four typical domains from the NH2 to the COOH end[67, 78]: 1) a 19 residues-long hydrophobic signal peptide; 2) a hydrophilic and glycosylated external ectodomain, approximatively 440 residues long; 3) an 22 residues-long transmembrane peptide; 4) an 85 residues long hydrophilic cytoplasmic domain. During the G mRNA translation, the first synthesized signal peptide traverses the endoplasmic reticulum towards the lumen, initiating a translocation process of the whole ectodomain. The translocation stops when the transmembrane peptide reaches the membrane where it stays anchored. A maturation process then cleaves the signal peptide and induces the glycosylation of the ectodomain. Finally, the mature protein appearing at the cell or virion surface is composed of the internal cytoplasmic domain, the transmembrane peptide and the glycosylated ectodomain protruding toward the outside.

The N mRNA translation is more simple, occuring totally in the cytoplasm, and producing a 450 amino-acid long polypeptide which is then phosphorylated at precise sites[23].

Figure 4 summarizes the numerous procaryotic and eucaryotic expression systems that were assayed.

D. Expression in Bacteria and Yeast

E. coli was extensively employed at the beginning, using several plasmid or virus vectors and inducible promotors, but was rapidly discarded because of its inability to glycosylate and cleave signal peptides. This required additional molecular biological tinkering to delete the signal sequence before expression. Although a heat inducible M13 phage promotor led to an important proportion of recombinant protein, approximatively 25% of the total proteins[36, 37], the resulting unglycosylated and denatured polypeptide showed absolutely no protective power in vivo.

In yeast, the 2 microns plasmid expressed a correctly glycosylated recombinant protein, but the production was too weak to envisage a genetic engineered vaccine[31, 37].

E. Expression in Animal Cells

In animal cells, several system were assayed, using either plasmid or viruses as vectors (Bovine papilloma virus, SV40 virus, adenovirus) but the production of the glycoprotein remained weak. Only a recent trial in adenovirus using three different promotors, allowed a substantial production of glycoprotein with a high protective power either by injection or by the oral route[52].

But the real first success was obtained with the Copenhagen strain of the vaccinia virus using the 7,5 Kd promotor[30]. The success of this system was both in expression and protective effects obtained by injection, by scarification, or by oral route[11, 37, 54, 76, 77]. Basically, a non-essential gene (here the TK gene), and a strong promotor (the 7,5 Kd) are isolated from the vaccinia virus genome. The rabies glycoprotein gene is placed under the control of the promotor and this construction is inserted into the middle of the TK gene on a convenient plasmid vector. After cotransfection of the vector with wild vaccinia genome in cells, double reciprocal recombination events using the TK gene flanking sequences insert the rabies glycoprotein in the middle of the vaccinia genome. This recombinant vaccinia virus is interesting because it is able to grow either in cell culture, or directly in animals to be vaccinated.

It was shown that laboratory animals injected intradermally or inoculated by the parenteral route with the recombinant virus develop high titer of virus neutralising antibodies and resistance to an intracerebral lethal challenge[76, 77]. Injection with the vaccinia wild type has no effect. Adult fox vaccinated by the oral route develop a neutralising antibody titer still detectable between 1-1.5 years after inoculation and are consistently protected against a lethal challenge[11, 22]. Since a fox lives approximately less than two years in the wild, this type of vaccination appeared particularly adapted. Oral vaccination campaigns by baits has been undertaken in Europe in the aim of eradicating the wild- (essentially fox-) rabies[47].

However, the Copenhagen strain of vaccinia virus that was initially used, was suspected of inducing side effects for human and animals, as previously observed during mass vaccination campaigns against smallpox [27, 28]. This was, from the beginning, a subject of controversy leading Copenhagen partisans to multiply the safety tests for an incredible number of animal species[22, 54], and Copenhagen detractors to look for new vaccinia strains or new poxviral vectors, with limited host range. Several alternatives were assayed. The New York Board of Health strain showing remarkably less side effects was used with a more powerful promotor, the 11KD[27]. However, its protective effect by the oral route appeared weak, perhaps because it is less invasive than the Copenhagen strain. The racoonpox virus was also tested to improve the host-specificity for the vaccine[28, 41]. Finally, the fowlpox virus, pathogen for poultry only, is still under development[62, 63]. Its interest as a vector is that it is unable to make a productive infection in non-permissive cells or non-permissive host. This greatly limits the possible side effects, notably a generalized infection to the vector in immunocompromised individuals. Despite this non-productive infection, fowlpox vector is able to express foreign genes, and particularly the rabies glycoprotein, at the surface of non permissive cells and thereby induces interesting levels of neutralizing antibodies by inolucation of several non permissive mammalian hosts.

More recently, expression of the glycoprotein gene in the baculovirus system was performed for CVS strain rabies virus[51] as well as for the rabies-related Mokola virus[14]. In this latter case, baculovirus appeared as a particularly convenient vector because, in contrast to rabies virus, Mokola virus grows efficiently in insect cells and was essentially isolated from insectivores in Africa. It was notably responsible for human encephalitis, death of domestic animals vaccinated against rabies, and for a limited epizooty in Zimbabwe[13]. The absence of cross protection by the classical rabies vaccines being verified in the laboratory, a specific vaccine against Mokola virus is necessary to protect exposed populations. The low growth efficiency of the virus in cell culture hypothesized the initial project of a classical cell culture vaccine and favours the search for an alternative baculovirus recombinant vaccine (unpublished results).

The technique used (figure 5) was basically similar to vaccinia virus. The Mokola glycoprotein gene was isolated, placed under the control of the polyhedrin promotor in a pEV55 transfer vector. Here again, the polyhedrin is not essential for the baculovirus and a cotransfection of the pEV55-GMok construct with the wild virus in SF9 insect cells, exchanges the polyhedrin gene with the Mokola glycoprotein in the recombinant virus.

When cells are infected with the recombinant virus, they synthesize a substantial quantity of a 56 kd recombinant protein similar both by electrophoresis and immunoblotting techniques to the native glycoprotein. In the presence of tunicamycin, which inhibits the N glycosylation, the apparent molecular weight of both recombinant and native protein are reduced to a similar extent, suggesting their effective glycosylation. By
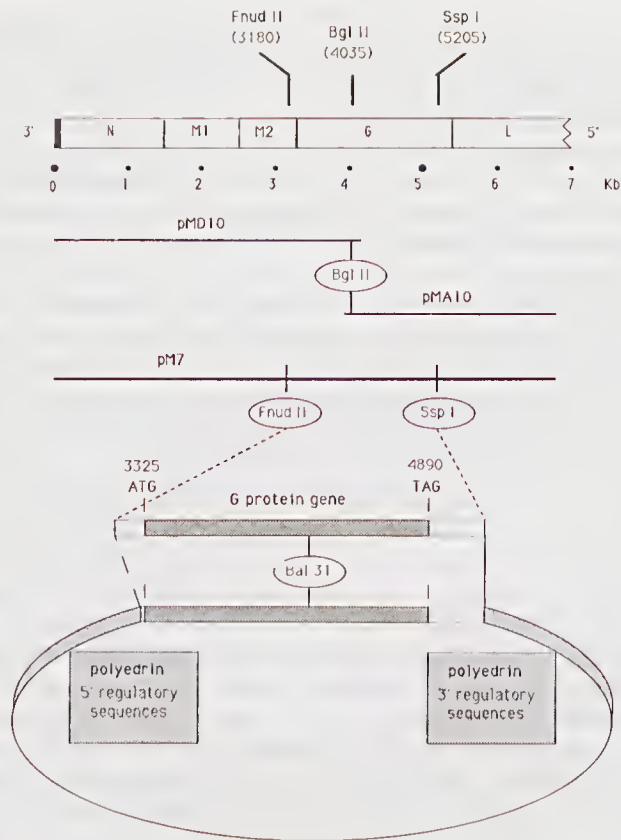
Figure 5 — Cloning of the coding sequence of the Mokola glycoprotein in a baculovirus vector. The cleavage sites of the restriction enzymes BglII, FnudII and SspI are indicated. pMD10 and pMA10 cDNAs are ligated by their common BglII site to form the pM7 cDNA. The FnudII-SspI internal fragment is excised, trimmed at the G protein NH2 side by the Bal31 exonuclease, and placed under the control of the polyedrin promotor in a pEV55 transfer vector.

treating the infected cells with a glycoprotein-specific monoclonal or a polyclonal antibody, the recombinant protein is shown to be expressed at the cell surface, as expected from its transmembrane nature. The protective power of the recombinant protein was analyzed. Mice injected intraperitonealy with insect cells expressing the recombinant glycoprotein resist an intracerebral challenge performed three weeks later. The protection is clearly due to the recombinant protein, since insect cells expressing the wild baculovirus are inefficient. It is specific for Mokola virus since mice remain sensitive to a challenge with the rabies CVS strain. The baculovirus recombinant Mokola glycoprotein is thus protective *in vivo* and constitutes the first experimental vaccine against a rabies-related virus with an excellent protective index. Additional purification and restructuration of the recombinant protein is now necessary before proposing a vaccine for human or veterinary use.

The expression of the N nucleoprotein gene was also performed in vaccinia[9, 41] and baculovirus sectors [8, 50, 53].

SciELO

VI. ARE THE CURRENT VACCINAL STRAINS ADAPTED TO THE CURRENT RABIES DISEASE?

1. The Vaccinal Strains

In contrast to the continual technical progresses in vaccine developments, very little was attempted to concomitantly adapt the vaccinal strains to rabies virus evolution. Consequently, the strains used for medical or veterinary vaccines, derive from isolates obtained between 50 and 100 years ago, following a complex history summarized in figure 6 and described in references[18, 35].

Most of them derive from the original Pasteur's isolate collected from a rabid cow in the suburb of Paris in 1882. They are therefore reminiscent of the dog-rabies that overran Western-Europe at the end of the 19th century and ignore the recent switch to the fox-rabies (see § II). The Pasteur's isolate was serially passaged on rabbit brains to give rise to the first "fixed" rabies strain (L. Pasteur strain) characterized by a constant and species-dependent incubation period. The L. Pasteur strain is still traditionally maintained at the Pasteur Institute of Paris (2074 passages) and has been adapted to cell culture. It was largely disseminated among the scientific and industrial communities to generate numerous "fixed" strains serving both for molecular studies and vaccine development. In 1940, the L. Pasteur strain penetrated the USA where it was maintained on rabbit brain and adapted to mouse brain by Karl Habel, resulting in the PM and CVS strains, respectively. It came back to Paris in 1965, after an obscure period in the Cepanzo of Buenos Aires, and gave rise to the PV strain.

Other classical strains alternatively derived from different isolates. In the USA, a rabid dog originated the SADs, SAG1, ERA and Vnukono32 strains, while a human isolate adapted to chick brain resulted in the Flury LEP and HEP strains. The Beijing31 and Kelev strains were isolated in China and Israel, respectively.

The letigimate question arising from figure 6 is the ability of current vaccinal strains to protect against the current rabies virus. Cross-protection studies have established that current rabies vaccines (serotype 1): 1) are globally efficient against members of the homologous serotype 1, although isolated cases of vaccination failures were reported in Africa[12]; 2) offer an imperfect protection dependent on the vaccinal strain used against serotypes 2 and 4[35]; 3) are ineffective against Mokola virus that constitutes the most divergent serotype 3[33].

This strongly plaids for the need for considerable efforts either to extend rabies vaccine potency towards a polyvalent Lyssavirus vaccine, or to propose new specific vaccines, particularly for viruses recognized as potentially dangerous for public health, such as Mokola virus[13]. As a logical first step in that goal, an intensive molecular epidemiological study of rabies virus appears urgent in order to appreciate the worldwide viral evolution and the antigen divergence at the genetic level.

2. Polymerase Chain Reaction (PCR) as an Alternative Tool for Diagnosis, Typing and Molecular Epidemiology of Rabies Virus

We have recently developed a very simple method based on PCR amplification of infected brain material which appears as a hopeful alternative for routine diagnosis, typing or precise molecular epidemiological studies[55]. At the epidemiological level, this method is advantageous to classical approaches based on antigenic differences with
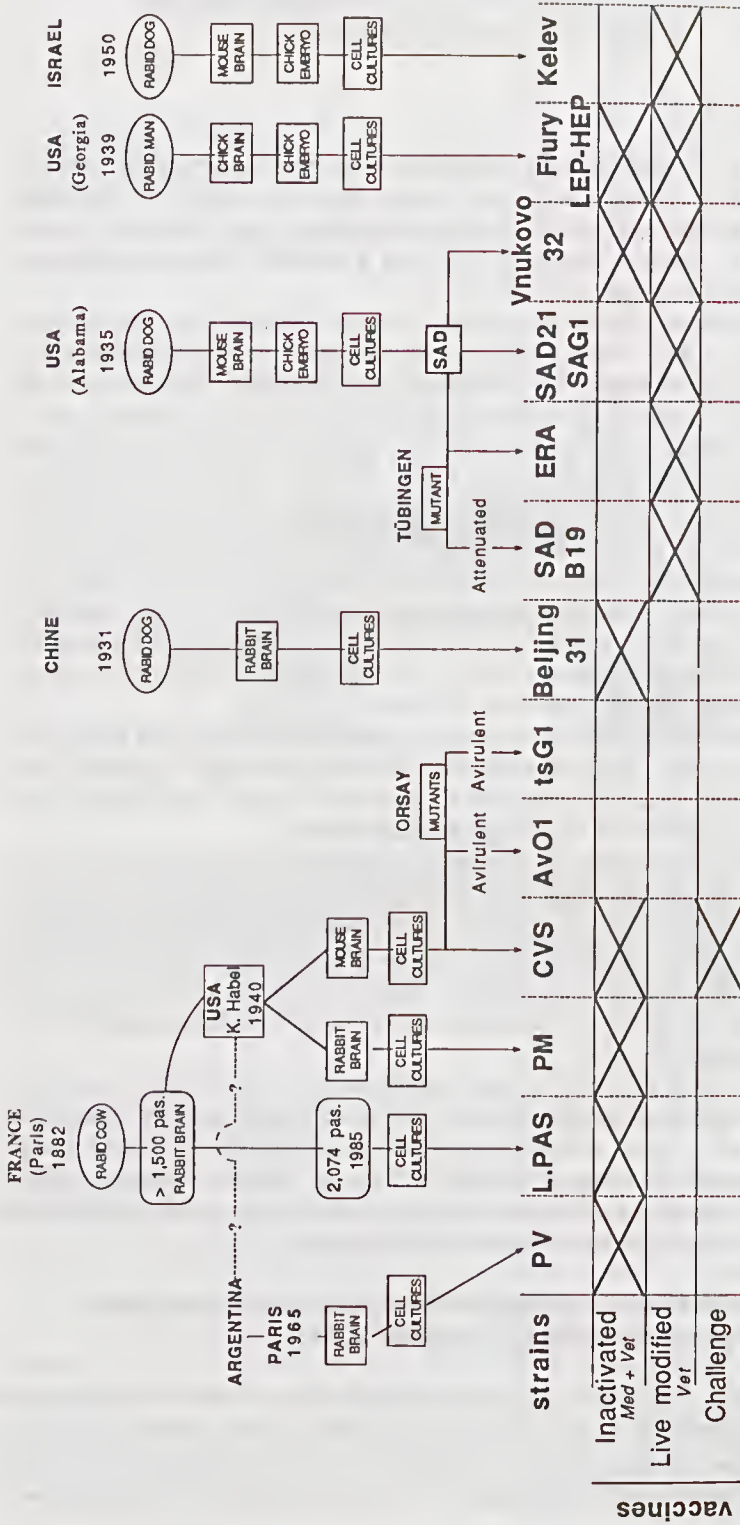
Figure 6 — Conspectus of the schematic history of the principal rabies virus strains used for vaccine development.

anti-N or -G monoclonal antibodies[24, 43, 57, 58, 72] because it does not require a previous cell culture adaptation setp, presumed to be possible source or selector of mutations. Using a disposable plastic pipette, brain samples are internally collected via the occipital foramen[6] or the retroorbital route[44] to avoid risks of contamination. Total RNA is extracted and reverse transcribed into cDNA which is consecutively amplified. The rabies specific primer used for reverse transcription can be one element of the couple used for amplification or different. One can amplify either genomic (minus sense) or messengers (plus sense) RNAs by selecting the convenient polarity for the reverse transcription primer. The amplified fragment can be progressively processed for:
- *diagnosis*, by electrophoresis on ethidium bromide agarose gel,
- *diagnosis or typing*, by differential hybridization with internal probes of divergent strains, either on dot or on Southern blots,
- *typing*, by restriction polymorphism with a limited panel of endonucleases,
- *molecular epidemiology*, by direct nucleotide sequencing of the fragment excised from agarose gel (Nusieve), without any additional purification, using the dideoxy technique (T7 Sequencing Kit, Pharmacia),
- *cloning and expression* in convenient vectors for fundamental or vaccinal purpose.

3. The Rabies ψ Pseudogene: The Best Clock of Evolution

The genomic areas targeted for amplification are different for diagnosis or epidemiological purposes. Conserved regions are more suitable for diagnosis which looks for minute quantities of viral sequences, whatever the infecting Lyssavirus. Highly variable regions are the more convenient for typing or molecular epidemiological studies where the important point is to find sensitive criteria to differentiate isolates.

From a comparison between rabies and Mokola genomes[15, 55, 68, 69, 70], which represent the two most divergent serotypes of the *Lyssavirus* genus, the N gene and the G-L intergenic regions were targeted for diagnosis and epidemiological studies, respectively (figure 7). The latter corresponds to a remnant protein gene, baptized ψ for pseudogene, placing the rabies virus in an intermediate position of unsegmented negative stranded RNA virus evolution, between the *Rhabdoviridae* and the *Paramyxoviridae* families[67, 69]. As a non protein coding region greatly susceptible to mutations, it is more likely to represent the natural evolution of the virus outside any external selective pressure and therefore the most suitable target for epidemiological studies.

4. Towards a Worldwide Molecular Epidemiological Study of Lyssaviruses

Using primers located in conserved places of the flanking G and L, the gene of worldwide fixed or wild Lyssaviruses has been successfully amplified and sequenced. The totality of the G and N genes of several isolates were also studied. In terms of divergence, the isolates rank in the same order, although at different rates, by considering either the coding G and N genes of immunological importance, or the non-coding pseudogene, respectively. This assesses "a posteriori" the significance of our rational approach of the viral divergence by the gene study, confirming this rapid method as particularly adapted for rabies epidemiological studies.

Although the results are still under exploitation, several major observations summarized in table I can be already shown (unpublished results):

1) The "fixed" strains used in vaccines form a dispersed group showing up to 18%
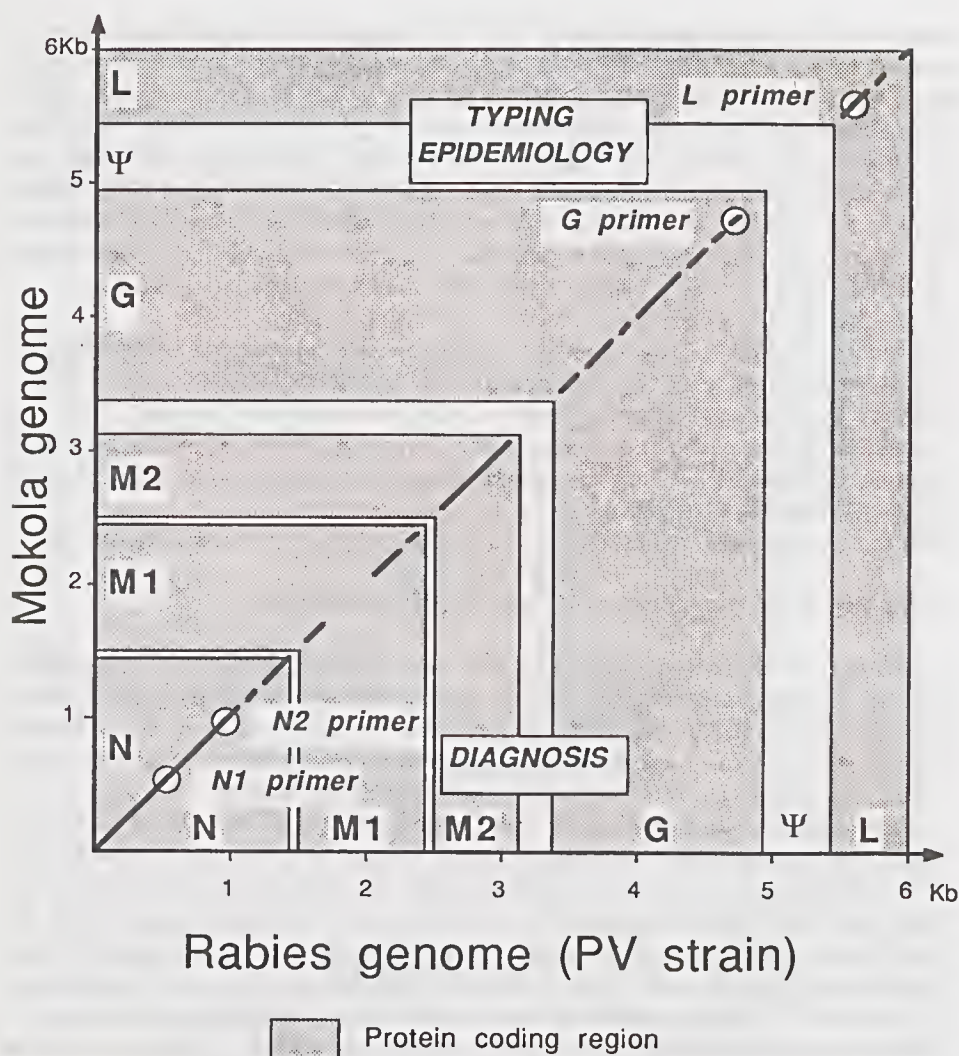
Figure 7 — Sequence comparison of the rabies (PV strain) and Mokola genome sequence. Diagonal lines indicate homologous areas. Regions convenient for diagnosis or typing-epidemiology purposes are noted.

internal divergence in the ψ gene (not shown).

2) The wild isolates of geographic proximity from relatively homogeneous groups. However, groups are substantially divergent from each other as well as from the vaccinal strains.

3) The West-African isolates, notably those suspected of vaccination failures, are consistently more divergent from the vaccinal strains than the French isolates against which the vaccines are clearly effective.

4) Bat isolates recently invading Europe or already present in Latin-America (Brasil, Guyana, not shown) exhibit a divergence almost as important as Mokola virus against which the rabies vaccines are clearly ineffective.

TABLE I

Divergence (%) between wild Lyssavirus groups and the classical vaccinal strain PV: France (12 isolates); West-Africa (Ivory Coast, Cameroon, Niger, Guinea, Marocco, etc...); European bat (France, Poland, Finland).

	ψgene/vaccine	Ggene/vaccine	ψ/group
France	14-15%	ND	2.5%
West-Africa	25-30%	15%	5%
Europ. bat	high	30%	ND
Mokola	high	40-45%	ND

VII. MAIN CHALLENGES FOR THE FUTURE

The recent progress in the molecular understanding of the rabies virus have not provided, so far, pertinent responses to the major enigmas of the disease itself. The reasons for the rabies virus neurotropy are still not understood, despite numerous hypotheses attempting to correlate the susceptibility of each cell type with the presence of specific receptors. Perhaps, the secret of neurotropy will reside in the study of more distal infection events, such as the influence of tissue-specific transcription factors on the mechanism of rabies genome expression. Furthermore, the mystery remains cloudy as to the nature of neuronal dysfunctions resulting in lethality, although their understanding would be a capital step towards an effective therapy.

Despite these numerous unsolved questions, the availability of any viral gene for studying and expression, is the most impressive contribution of the last ten years. In the near future, examination of viral isolates from various regions of the world will permit an evaluation of the spacio-temporal evolution of the virus and the influence of the host. It will be interesting to understand the basis of the cross-protection at the sequence level, in order to decipher whether the current vaccinal strains are sufficient for animal and human health, or if additional specific vaccines are required. These could be performed by DNA-recombinant techniques, taken into account the progress in rabies virus immunology. For example, synthetic structures carrying a recombinant glycoprotein(s) anchored on their surface, and containing a recombinant nucleoprotein(s) (or T-peptides) should be promising in the goal of a genetic engineering vaccine against all (or most of) the members of the *Lyssavirus genus*.

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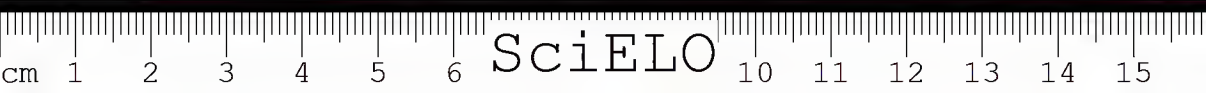
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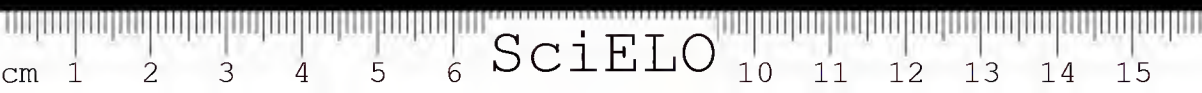
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INSERTION OF HETEROLOGOUS EPITOPES IN *SALMONELLA* FLAGELLIN

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INTRODUCTION

Strains of *Salmonella* with non-reverting mutations in the common aromatic biosynthesis pathway have been constructed[4] and found to be attenuated and highly effective as live vaccine in several animal models[12, 14]. The lack of virulence is probably associated with their requirement for paraminobenzoic acid, not available in mammalian tissues. Therefore, aromatic-dependent *Salmonella* multiply for only a few generations in the host; however, they persist, as live bacteria, in the liver and spleen of mice for weeks. Such persistence accounts for a strong immune response by the host, providing long lasting protection against challenge with homologous strains.

Aromatic-dependent *Salmonella* strains have been successfully used to expose heterologous antigens to the immune system. An aromatic-dependent *S.dublin* strain carrying a plasmid harboring the gene for the B subunit of heat-labile *E. coli* enterotoxin was able to evoke serum IgG and mucosal IgA antibodies to LT-B after oral administration to Balb/c mice[2]. Similarly, an aroA strain of *S. typhimurium* carrying a plasmid specifying constitutive expression of beta-galactosidase induced cellular and humoral responses to beta-galactosidase, as measured by footpad swelling test and ELISA[1].

The immune response to a cloned antigen may be enough to cause protection. Indeed, an aroA strain of *S.typhimurium* carrying a plasmid determining production of the M protein of a *Streptococcus pyogenes* strain was able to protect against intraperitoneal

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challenge with 100 LD_{50} *S. pyogenes* after oral immunization of Balb/c mice[11]. Vaccination of Balb/c mice with aroA *Salmonella typhimurium* expressing the circumsporozoite protein from *Plasmodium berghei* conferred protection against rodent malaria[13].

Since the immune reponse directed to some antigens resides in short amino acid sequences (linear epitopes), it is possible to engineer recombinant bacterial proteins carrying foreign antigenic determinants. When expressed by live vaccine strains of *Salmonella*, such hybrid proteins are likely to evoke immune responses to the inserted peptide.

Flagellin, the protein that makes up the bacterial flagellar filament has been considered an attractive target for epitope insertions, since there is great variation in its amfnoacid composition, a fact revealed by the number of flagellar serotypes in nature. The cloning and sequencing of four flagellin genes of different serotypes from *Salmonella* showed near identity between the genes for ca. 150bp at each end of the genes, and increasing diversity toward the central region, where no more than about 30% homology in aminoacid sequence was detected for any pair-wise comparison[16]. A previously identified epitope of flagellar antigen i was located with segment IV[6]. Mutants of a cloned flagellin H1-d gene with altered antigenicity showed a point mutation or deletions within segment IV[9]. Therefore, the central, hypervariable region is thought to specify antigenic determinants present at the flagellar filament's surface. Insertion of epitope-specifying oligonucleotides in that region could result in exposition of a foreign epitope as a flagellar antigenic determinant.

MATERIALS AND METHODS

Plasmid pLS402 is a pBR322 with a 3.8kb insert from *S.muenchen* harboring the H1-d flagellin gene (provided to us by Dr. T.M. Joys). Plasmid pLS408 was derived from pLS402 by cloning the EcoRI insert in pUC19 and deleting a 48bp EcoRV fragment, inside the hypervariable region, generating a single EcoRV site (GAT ATC) in between two codong of the H1-d gene. Oligonucleotides were designed to allow in-frame insertion at the EcoRV site of plasmid pLS408. They were purchased from Operon Biotechnologies Inc., California.

Strain CL447 is a C600-derivative with a deletion at its single flagellin gene, hag[18]. It is non-motile but becomes motile when given plasmids pLS402 or pLS408. Strain LB5000 is an *S. typhimurium* strain r-m+ used to modify plasmids from *E. coli* to prevent restriction in the live vaccine strain SL5928. SL5928 is an *S. dublin* aroA strain with its single flagellin gene replaced by a gene inactived by the insertion of transposon Tn10.

All molecular manipulations were carried out according to Maniatis et al[7]. Protein analysis was made by western-blotting[15] and the immune responses followed by ELISA[3].

RESULTS

Epitope CTP3 of cholera toxin subunit b as insert

The CTP3 epitope comprises residues 50-64 of the B subunit of cholera toxin[5]. Oligonucleotides specifying this sequence were synthesized with a 15bp overlapping region, leaving 5' overhangs to be repaired by Klenow fragment. The resulting double-

stranded segment was blunt-end ligated to EcoRV-digested pLS408 and transformed into CL447 strain. Plasmids with the insert in correct orientation (as shown by sequencing) were transferred to LB5000 strain and from this strain to SL5928. Hybrid flagellin genes conferred motility on the flagellin-negative recipients (*E. coli* CL447 and *S. dublin* SL5928). Western blotting of lysed bacteria or isolated flagellin showed a single band of the expected size, binding both rabbit anti-d antigen and monoclonal anti-CTP3 antibody. Exposure of CTP3 at the surface of the flagella was shown by immobilization of the bacteria by monoclonal CTP3 antibody and also by immunogold labelling. Live-vaccine strain of *S.dublin* expressing the chimeric flagellin gene was given to C57BL/6 mice, at three weekly intervals, i.p. (5 x 106 bact/mouse) and their sera analysed by ELISA. Sera from all animals reacted with CTP3 peptide and whole cholera toxin [8].

Epitopes of hepatitis B surface antigen as inserts

Epitopes from the surface antigen (S122-137) and from the pre-S2 region (120-145) were inserted in flagellin essentially by the same procedure employed for CTP3 insertion. Results are summarized in table I. Recombinant plasmids with the oligonucleotides inserted in correct orientation (pLS414 for S122-137 and pLS428 for 120-145)

TABLE I

Insertion of oligonucleotides specifying hepatitis B surface epitope (residues 122-137) in vector pLS408. Arrows outside boxes indicate direction of transcription of flagellin gene H1-d. Boxes represent inframe insertions, with the two possible orientations indicated by arrows.

Structure	Plasmid	Motility	Western-blotting	
			anti-peptide	anti-flagellin
—[→]—→	pLS414	-	+	+
—[←]—→	pLS503	+	-	+
—[←][←][←]—→	pLS504	+	-	+
—[→][←]—→	pLS413	+	+	+

originated non-motile *E. coli* or *S. dublin* clones. By western-blotting, a band binding anti-flagellar antigen d and anti-peptide antibodies was detected in both cases, but no flagella were seen by electron microscopy. Insertion of S122-137-specifying oligonucleotide in reverse orientation produced functional flagella, even when 3 copies of the sequence were inserted (48 aminoacids). One recombinant plasmid, pLS413, showed two copies of the oligonucleotides, one of them in correct orientation, and originated functional flagella. Therefore, it was possible to compare the immune response to the hepatitis epitope when present at the flagellar filament's surface (pLS413) or intracellularly, as flagellin (pLS414). Both constructs, when expressed by the live vaccine strain SL5928, evoked antibodies to the inserted peptide to the same level[17].

M protein epitope as insert

The *Streptococcus pyogenes* type-5 M protein comprising the 16 amino terminal residues of the mature protein was inserted in flagellin, since using the whole protein expressed in *Salmonella* to immunize mice p.o. conferred protection against challenge with the *Streptococcus* strain. Immunization with the whole protein, while effective to confer protection, has been hampered by the fact that such protein presents epitopes that cross-react with human heart tissue. The epitope chosen for insertion in flagellin, has been recently characterized as protective, and unable to evoke auto-immune responses[10].

Recombinant plasmids showing the oligonucleotides in correct orientation originated functional flagella, similarly to what has previously been observed for the cholera toxin epitope insertion. Exposition of the epitope at the flagellar filament's surface was revealed by immobilization and immunogold labelling. Mice immunized i.p. with live SL5928 expressing the chimeric flagellin gene and rabbits immunized i.m. with the same strain, formalin-fixed, made antibodies to the M protein peptide, with opsonizing activity (Table II).

Table III summarizes the immune response of all epitopes described herein.

TABLE II

Summary of immune responses of mice vaccinated with strain SL5928 expressing flagellin with an M5 insert or given the same strain expressing flagellin with no heterologous epitopes.

	Elisa Titres (*)		Opsonization
Week	Peptide	*S. dublin*	(**)
Mice immunized with strain SL5928 expressing plasmid pL435 (M5 insert in flagellin)			
0	<100	<100	0
2	200	3,200	6
4	800	12,800	52
6(***)	6,400	25,600	96
Mice immunized with strain SL5928 expressing plasmid pL408 (no insert in flagellin)			
0	<100	<100	0
6(***)	<100	12,800	0

(*) Titres of pooled sera from 5 mice in ELISA test with synthetic M5 peptide or whole bacteria (SL5928) as test antigens.

(**) Percent of human neutrophila with one or more associated streptococci.

(***) Two weeks after the vaccine dose, the mice were challenged by i.p. injection of 100 L.D.50 streptococci. None of the mice given the live-vaccine strain expressing pLS408 survived. Only one of the 5 mice given the hybrid live-vaccine strain died.

TABLE III

Summary of immune responses for all epitopes described in this paper.

Epitope (residues)	Plasmid	Motility	species	doses	no. of doses	route	ELISA (+/tested)
Cholera toxin subunit B (50-64)	pLS411	+	C57BL/6	$5x10^6$, live	x3	i.p.	5/5
			C57BL/6	$5x10^6$, killed	x3	i.p.	5/5
			C57BL/6	10^9, live	x3	p.o.	0/5
Hepatitis B surface protein S (122-137)	pLS414	-	rabbit	10^9, live	x5	i.m.	2/2
			Balb/cJ	$5x10^8$, live	x4	p.o.	10/10
			guinea pigs	10^9, live	x4	p.o.	3/3
Pre-S2 (120-145)	pLS428	-	rabbit	10^9, live	x5	i.m.	2/2
			B10/BR	$5x10^8$, live	x4	p.o.	10/10
			guinea pigs	10^9, live	x4	p.o.	3/3
S (122-137)	pLS413	+	rabbit	10^9, live	x5	i.m.	2/2
			Balb/cJ	$5x10^8$, live	x4	p.o.	10/10
			guinea pigs	10^9, live	x4	p.o.	3/3
S. pyogenes type-5 M protein (42-57)	pLS435	+	rabbit	10^8, killed	x3	i.m.	2/2
			Balb/cJ	$5x10^6$, live	x3	i.m.	5/5
			Balb/cJ	10^9, live	x3	p.o.	0/5

CONCLUSIONS

The central region of flagellin gene H1-d seems appropriate for insertion of epitope-specifying oligonucleotides, since humoral immune responses have been detected against all epitopes inserted so far. The generation of immune responses to the foreign peptide and to flagellin is not dependent upon flagellar function, a fact that greatly extends the probable usefulness of the system. Our results with the hepatitis B surface antigen indicate that it might be possible to insert several epitopes without preventing flagellar assembly and function. However, at the moment we do not know the features of an aminoacid sequence that allow normal flagellar function. Immunization with live-vaccine strains of *Salmonella* presents several advantages: effective immunization by oral route, induction of cellular and humoral immune responses as well as mucosal immunity, and safety, due to the non-reverting aromatic mutation. Experiments are under way to determine the maximum size of insertion compatible with flagellar function, the possibilities of using other sites in the flagellin molecule for insertion of epitopes, and the induction of cellular immunity to a heterologous epitope inserted in flagellin.

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THE IMPACT OF RECOMBINANT DNA ON THE CONTROL OF ANIMAL HEALTH

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INTRODUCTION

Although there are at present a large number of different products for controlling diseases and improving productivity of livestock approximately one hundred billion dollars is lost annually to the world economy due to death losses, treatment costs or reduced productivity. The advent of recombinant DNA techniques has the potential to develop a variety of new diagnostic procedures as well as new vaccination strategies to help in the campaigns against economically important diseases of livestock.

Foot-and-mouth disease virus (FMDV) will be adopted to exemplify the impact of recombinant DNA of the new developments.

The causative agent of foot-and-mouth disease (FMD) is an aphthovirus belonging to the family *picornaviridae*. The virion is icosahedral, without envelope, of about 25 nm of diameter and consists of 60 copies each of 4 coat protein VP_1, VP_2, VP_3 and VP_4. VP_1 is the only structural polypeptide that, when purified and injected into cattle, is capable of inducing neutralizing antibodies [1,2,3]. The viral genome consists of a single-stranded RNA of approximately 8000 nucleotides. The viral RNA is infectious and serves as mRNA. Upon translation a polyprotein is produced which is subsequently cleaved into a series of intermediate precursors which are further processed to give the mature non-capsid and capsid proteins.

This system provides an excellent model to explore many of the alternative possibilities of diagnosis and vaccines because: – The virus has a relatively simple structure compared to other agents; – Foot-and-mouth disease is obviously one of the major dis-

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eases of domestic farm animals; – It is an acute disease characterized by vesicular lesions which lead to significant productivity losses; – After the acute phase of the disease, the virus established inaparent persistent infections which constitute an economic burden to cattle exporters because of the restrictions placed on the export of FMDV-positive animals or animal products to disease-free areas; – Therefore the disease causes one of the major economic impacts in livestock industry. It is estimated that in South American countries, at least 25% of the reduction in livestock productivity is due to this disease, causing losses and expenses of 500 million dollars annually; – Variability of the virus during replication leads to variants often not neutralized by the vaccines in use. Variability is critical during persistent infections[4,5]; – The potential of new biotechnological strategies should be evaluated in order to develop:

a) highly sensitive and specific methods for detection of carrier animals;
b) precise techniques for the characterization of FMDV to establish the similarities between field and vaccine strains;
c) more effective vaccines: Conventional vaccines have been available since 1938 and in addition, 800 million doses are used annually, so that efficacy of new alternatives will have a good point of reference.

DIAGNOSTIC PROBLEM IN SUSPECTED APHTHOVIRUS DISEASE

The identification of carrier animals infected with FMDV is usually made by the isolation of FMDV of material obtained from esophageal-pharyngeal (OP) fluids, in tissue culture [6,7,8,9]. Moreover, screening for antibodies against the FMDV-infection associated antigen (VIAA)[10-13], the FMDV RNA polymerase [14,15], in animal sera is also carried out routinely. These tests are internationally accepted for import/export testing and as an epidemiological tool to determine the spread of FMD in animal populations. In practice, these methods do not always yield conclusive results. No clear correlations could be established between these methods suggesting technical problems with the OP fluid sampling and/or the tests used to detect the anti-VIAA antibodies. Virus isolation procedures appear to be successful in the acute phase of the disease with extensive virus replication, however persistent virus from OP samples is only occasionally recovered during the whole carrier state.

The identification of serum antibodies against the VIA antigen by the immunodiffusion test in agarose gels (IDAG)[10-12] is not sensitive enough. Attempts to increase the sensitivity through an enzyme-linked immunosorbent assay (ELISA)[16] raised questions with regard to the development of VIAA antibodies in cattle vaccinated and revaccinated with vaccines produced in baby hamster kidney (BHK) cells, containing high concentrations of non-purified FMDV-antigens. Since the VIA antigen used for these tests is only partially purified from BHK-infected cells, traces either of FMDV capsid polypeptides or BHK antigens may be recognized by sera of animals immunized with BHK-produced vaccines, leading to false positive tests.

Another significant limitation of these methods is the requirement of a high security laboratory unit for handling FMDV, which constitutes a problem, especially in FMD-free areas.

INTRODUCTION OF NEW DETECTION STRATEGIES

So far we have chosen two approaches to potentially overcome the mentioned limita-

tions. The first approach was based on the use of new diagnostic approaches for the detection of FMDV in OP samples which included: a) molecular nucleic acid hybridization techniques (dot blot, northern blot or in situ hybridization), using cloned viral DNA as a diagnostic reagent for detection of the viral RNA sequences and b) amplification of specific viral genomic fragments using the polymerase chain reaction (PCR). The second approach was to develop highly sensitive and specific methods to detect in sera of suspected carrier animals the presence of antibodies against FMDV-nonstructural antigens, including others than the VIAA traditionally used. Immunochemical techniques (ELISA, immunodot and immunoblotting) using as serological probes highly purified bioengineered VIAA as well as other bioengineered nonstructural antigens were attempted.

One major prerequisite for the introduction of these strategies as diagnostic tools in suspected persistent aphthovirus infections was the molecular cloning of the aphthovirus RNA genome in order to get a complementary DNA (cDNA) as a probe for the FMDV genome, to be used in molecular hybridization tests. Besides, the cloned cDNA is further used to obtain the bioengineered serological probes, by expressing defined genomic regions, coding for the different nonstructural proteins, in *E. coli.*

Intact 35S viral RNA was prepared, copied into cDNA using oligo (dT) as a primer and reverse transcriptase. The cDNA was then fractionated on an alkaline sucrose gradient. The largest cDNA fractions were further inserted through standard techniques into a bacterial plasmid.

Clones covering the whole coding region of the genome were obtained.

CLONED APHTHOVIRUS cDNA AS A DIAGNOSTIC REAGENT

If one addresses nucleic acid hybridization as a diagnostic tool for the detection of aphthovirus carriers a major prerequisite is the demonstration that the radioactively labeled cloned cDNA detects specifically viral nucleic acids and does not hybridize to total cell RNA. When radioactively labeled cloned aphthovirus cDNA corresponding to the genomic region coding for the viral polymerase was hybridized to RNA immobilized on nitrocellulose paper, extracted from OP samples of control or experimentally persistently-infected cattle, specific hybridization was found only for FMDV-infected tissue RNA and not for total tissue RNA. The minimal detection level under high stringency conditions, that we observed was 1 pg of genome FMDV-RNA. Thus, the use of cloned cDNA provides a valuable diagnostic tool for detection of carrier cattle.

A main advantage of the nucleic acid hybridization approach in suspected FMDV infections is given by the fact, that detection of different aphthovirus serotypes is possible by using just one cloned cDNA as a probe. The molecular basis for this finding is given by the high degree of nucleic acid sequence homology among the different serotypes for example in the genomic region corresponding to the viral polymerase. The exact typing of the implicated strain could also be carried out by using serotype-specific DNA fragments corresponding to the viral genome encoding for the viral coat proteins.

Hybridization directly in fixed tissues is another interesting approach to be considered. It is a worthwhile tool especially for studies of viral pathogenicity, since it permits the direct identification of the virus-infected target cells and an estimate of the proportion of cells carrying the viral genome within a section.

Other probes for veterinary applications include: Pseudorabies, Bovine herpes virus I, African swine fever, Bluetongue, Marek's disease, Anaplasma marginale, Babesia

and Enterotoxigenic *E. coli.*

AMPLIFICATION OF SPECIFIC APHTHOVIRUS GENOMIC RNA SEGMENTS

A major breakthrough for diagnostic procedures is given by the polymerase chain reaction (PCR)[17]. It consists on the capacity to amplify specific segments of DNA through an *in vitro* enzymatic synthesis of a specific DNA fragment using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. This synthesis is achieved through a repetitive series of cycles involving denaturation, primer annealing and extension of annealed primers.

Since primer extension products can serve as templates in the next cycle, there is an exponential accumulation of specific fragments whose termini are defined by the 5' ends of the primers, so that 20 cycles can yield about one million fold amplification.

For the detection of viral RNA it is necessary to first obtain a cDNA copy by the reverse transcription, which is then used as target DNA.

We have applied the PCR method to the amplification of defined segments from the genomic region coding for the structural polypeptides of partially purified FMDV-RNA of serotype O_1. Primers were chosen from highly conserved regions and purified. Successful amplification of fragments of 850 bp covering the whole VP_1 region and of fragments of up to 2000 bp covering the genomic region coding for the capsid polypeptides VP_3, VP_2 and VP_1, were obtained. In contrast, amplification of segments of more than about 2000 bp normally resulted in several low molecular weight bands and only rarely gave a specific reaction.

The specificity of the amplified material was confirmed by restriction enzyme analysis of amplification products of the correct molecular weight, and is being further confirmed by sequencing after subcloning into SP64.

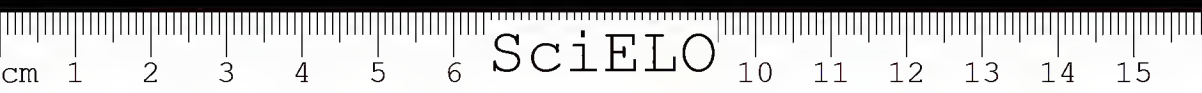
Our future goal is to amplify FMDV-RNA segments from biological specimens such as cells persistently-infected with FMDV and biopsies from experimentally-infected animals. The method should be adequate for rapid diagnosis of FMD, even when minimal quantities of virus are present.

A great advantage of this extremely rapid and sensitive method for the detection of FMD is that nucleotide sequence information can be obtained directly from clinical samples without the prior need for virus amplification in cell culture, thus avoiding the possibility of *in vitro* induced selection and modification of virus populations.

DETECTION OF FMDV-ANTIBODIES IN SERA OF PERSISTENTLY-INFECTED ANIMALS

In order to overcome the limitations mentioned above, we have attempted the use of immunochemical techniques using bioengineered VIAA and other bioengineered aphthovirus nonstructural antigens.

Compared to the classical VIAA obtained from infected cell cultures, the use of bioengineered antigens offers several advantages: safety, does not require a high security laboratory for its production; yields, higher yields can be obtained in bacteria than in infected cells; and simplicity and costs, bacterial cultures require less space, time and effort than eukaryotic cell cultures and are significantly less expensive. In addition, sero-

logical probes can be prepared for nonstructural antigens which can not be purified from infected cells, so that the most suited nonstructural antigens of potential diagnostic significance can now be identified by studying the antibody response of the host to the different aphthovirus nonstructural proteins during different stages of persistence.

To obtain the most adequate bioengineered VIAA for use as a serological probe, we constructed a bacterial expression vector (carrying the inducible PL promoter of bacteriophage Lambda in front of a consensus Shine Dalgarno sequence) coding for the complete VIA antigen. The strategy was designed so that the polypeptide expressed would only have one additional amino acid the N-terminal methionine[18].

The recombinant polypeptide was expressed and the soluble fraction from the bacterial extracts was further purified by chromatography over a series of phosphocellulose column and poly(U)-Sepharose column. The identity of the purified protein was confirmed by immunoblotting with sera of convalescent animals. We further analyzed the potential of the bioengineered native VIAA to detect specifically anti-VIAA antibodies in sera of experimentally persistently-infected cattle.

Analysis by immunoblotting indicated that antibody binding to the bioengineered native VIA polypeptide was constant and it was detected in sera from infected cattle as early as at 7 dpi, giving a peak at 6 weeks postinoculation with the intensity of the bands decreasing gradually with time after infection, and being still positive at DPI 644, time by which all detections were negative by the classical IDAG test. In contrast to the results obtained previously[16], under the conditions used, no detectable reaction was obtained with sera from control cattle (obtained from FMD-free regions) and/or vaccinated cattle.

The induction of antibodies to other nonstructural proteins was analyzed by using a set of bioengineered antigens obtained by expressing defined regions of the genome in *E. coli*, as serological probes[19]. Western blot analysis with sera of experimentally infected cattle shows that although all antigens gave a positive reaction, antigens 3A and 3B gave the highest signal/noise positive detection when tested either with sera from convalescent or late persistently-infected cattle. Antibody induction to antigens 3A and 3B shows a peak 5 weeks later than that obtained for the VIA antigen, decreasing then gradually with the increasing weeks postinoculation, but to a lesser extent than for the VIA antigen. Again, none of the sera of FMD-free regions or from vaccinated cattle gave a positive reaction.

Our ultimate goal is to develop a rapid and simple assay for the detection of carrier animals. The use of additional nonstructural antigens together with immunoblotting, could provide a method for simultaneously screening a single serum for the presence of antibodies against multiple antigens which may fluctuate during the course of the disease.

Examples of other animals diseases which have immunochemical diagnostic assays include: Bluetongue, Salmonella, Pseudorabies, Parvovirus, etc.

MOLECULAR CHARACTERIZATION OF APHTHOVIRUS STRAINS

Control of FMDV is complicated by the occurrence of the virus in 7 serotypes. The European types O, A and C, also present in South America, the South African Territorie type Sat_1, Sat_2 and Sat_3 and the Asiatic type $Asia_1$. In addition, over 60 known subtypes resulted from variation within each serotype, with little cross reactivity among them. Therefore, the constant monitoring of field strains is essential to ensure that vaccines in current use are effective, i.e., contain strains sufficiently similar to those circulating in the field.

Several techniques for the analysis of nucleic acids and proteins are being used. Such techniques include nucleic acid analysis through RNA fingerprinting, and sequencing as well as protein analysis through SDS-PAGE, isoelectrofocusing, two-dimensional gel electrophoresis, etc. These biochemical methods together with the development of monoclonal antibodies and their use in ELISA tests provide ideal tools for the precise biochemical and antigenic characterization of active, evolving, vaccine and laboratory strains.

We are intensively involved in the biochemical and serological characterization of field and vaccine strains.

Fingerprinting is used routinely for comparing closely related strains, and so: evaluate genetic stability of strains during vaccine production, establish possible vaccine origin of field outbreak and monitor origin behaviour and fate of new strains. Examples of outbreaks caused by viruses of serotype C_3 which showed minor serological variations from the prototype strain C_3 Resende as well as very similar fingerprinting patterns, occurred in Argentina between 1982-1984[20]. The similarity of the isolated strains with that of the prototype strain, was taken as an indication that these strains had not circulated for long in the field and that they had been freshly introduced through escape of a laboratory or an incomplete inactivated vaccine.

The significance of the evolution of viruses in the field can be exemplified by the characterization of strains isolated from an outbreak caused by strains serologically identified as a C_3 variant which took place in Argentina during 1984 and up to 1986. This outbreak could not be controlled by the vaccines in use containing the prototype strain C_3 Resende [20]. Representatives of this outbreak were isolated, studied and later included in the vaccines in order to finally control the outbreak. Fingerprinting showed them to be significantly different from the prototype strain C_3 Resende. Moreover, relevant changes in the structural polypeptides could also be shown. Although the epidemiological origin of these strains has not been traced, it is possible that in endemic regions viruses could be maintained under conditions which they are replicating such as occurs in persistently-infected cattle. We have demonstrated that during persistence, variability is very critical. During this time, rapid evolution of viruses occurs. We found a clear cut relationship between the degree of genomic variations and the number of days postinoculation, indicating a gradual and progressive evolution of the strains during the persistent stage. In addition, we described a decreased reactivity of FMDV persisting at 63 dpi to a set of neutralizing monoclonal antibodies. Sequencing data also showed the accumulation of mutations which represented $2x10^{-2}$ substitutions/nucleotide/year, 60% of the changes resulted in changes of amino acids. Some of the changes occurred in amino acid residues 40-50 and 135-153, considered to be important immunological domains [5]. This fact demonstrates the high risk for the animals and for those susceptible hosts in the surroundings.

PROSPECTIVES FOR THE DEVELOPMENT OF NEW VACCINES

Although vaccination to prevent virus infections has been practiced for over two centuries, the process has changed relatively little since the time of Jenner. Most of the immunogens of choice used today are still killed or attenuated viruses. Recent attention has focused upon the design and production of vaccines consisting of non-viable (non-replicating) and non-infectious portions of the pathogenic agent that are still capable of eliciting a protective immune response.

Potential vaccine designs include:

I. *Subunit vaccines*
   - a) Expression
     - Bacteria
       - Eukaryotic systems:
         - Yeast
         - Poxvirus
         - Baculovirus
   - b) Synthetic peptides

II. *Attenuation* by direct gene manipulation

III. *Anti-idiotypes*

IV. *Antisense:* Infectious resistant cells

V. *Complementation* of a virus deficient strain in cells constitutively producing the defficient protein

VI. *Anti-cell receptor:* antiviral

SUBUNIT VACCINES

This approach became possible when some structural features necessary for eliciting a good immune response were identified:

- the neutralizing activity is largely confined to VP_1; VP_1 isolated and used as a vaccine elicits neutralizing antibody responses and protects cattle and swine from infections;
- the neutralizing activity was generated by fragments obtained by cyanogen bromide or enzymatically, spanning the regions corresponding to amino acid residues 145-154 and 201-213.

The aphthovirus structural gene coding for VP_1 was expressed in *E. coli.* Although a significant level of expression was obtained, the protein produced, evoked no neutralizing antibody response up to 30 days postrevaccination.

These results are not surprising considering that the isolated protein is weakly immunogenic possessing less than 0,1% of the activity of the virus particle [21]. It becomes always more evident that VP_1 in the virion adopts a conformation which is highly dependent from a substantial interaction with the other structural proteins [22].

In the case of other infectious agents, expressed in prokaryotic systems, the first outstanding example of a genetically engineered bacterial vaccine which became commercially available was against the somatic pili of enterotoxigenic *E. coli* strains, the cause of diarrheal diseases in young livestock [23].

A recently developed baculovirus expression system utilizes the highly expressed and regulated *Autographa californica* nuclear polyhedrosis virus polyhedrin promoter modified for the insertion of foreign genes [24]. Encouraging results were obtained expressing bluetongue virus neutralization antigens VP_2 and VP_3 [25], pseudorabies gp 50 [26], Rift Valley fever virus antigens G-1 and G-2 [27] and simian rotavirus SA11 capsid antigen VP_6 [28].

SYNTHETIC PEPTIDES

It is fortunate that FMDV has been one of the most studied with regard to immuniza-

tion with peptide antigen and the results suggest that there could be a practical outcome to the work.

Several approaches predicted amino acid residues 144-159 and to a lesser degree 200-213 of VP_1 as good candidates for eliciting a neutralizing response [29-30].

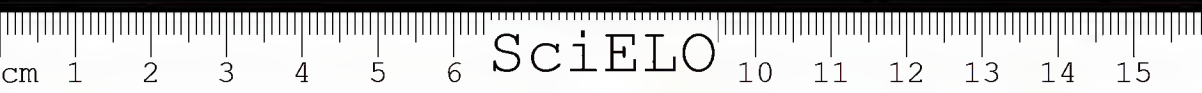
According to these predictions we synthesized a series of peptides, covering various regions of the polypeptides, corresponding to the sequence of serotype O_1 Kaufbeuren, by using the solid phase Merriefield process. The peptides were linked to keyhole limpet haemocyanin and tested for immunogenicity in guinea pigs. Synthetic peptides representing each of the two potential immunogenic regions of VP_1 induced high levels of antibodies which recognized intact virus, but only residues 140-160 were protective. Despite the optimal results obtained in guinea pigs with the peptide corresponding to residue 140-160, when this peptide was inoculated in cattle, very low levels of neutralizing antibodies were obtained even after revaccination[31]. The potential importance of sequences 200-213 in enhancing the response of sequences 141-160 was recently suggested [32]. Moreover, a very encouraging result was obtained using tandem peptide sequences fused to bacterial proteins. Already protection of cattle and swine has been achieved with one injection of as little as 40μg of peptide. However, only limited animals were used in the trial [33].

A key issue in the field of peptide vaccines is the potency of the adjuvant. Several new adjuvants have been tried, including the use of what has been termed immunostimulating complex (ISCOM), in which the virus proteins are incorporated into cagelike structures by complexing them with saponin, a plant glycoside.

In the case of other animal infectious agents, encouraging results were obtained with vaccines against rabies and rotavirus [34-35].

LIVE ATTENUATED VACCINES

Live viral vaccines offer significant advantages over other types of viral vaccines, namely, induction of more effective local immunity, longer duration of immunity and spread of the vaccine strain among susceptible hosts within the population. Such vaccines, however, are at present only of limited use due to a) the possibility that an attenuated virus strain could revert to its more pathogenic form; b) potential susceptibility of hosts other than the one for which the vaccine is attenuated and c) an optimum equilibrium between pathogenicity and immunogenicity is not always obtained. Such limitations could not be overcome in the late fifties when these attenuated vaccines were developed [36].

The observation for several picornaviruses that viable virus can be rescued from cloned cDNA was an essential breakthrough to study the genetic determinants of attenuation and to construct safe attenuated vaccines. Infectious DNA can be specifically modified to obtain attenuated strains which can replicate and retain antigenic identity without propensity for virulence. Moreover, once a stable attenuated strain is identified or generated through adequate alterations of an infectious clone, and provided that the genetic determinants of attenuation are not located in the immunogenic regions, one can extend the attenuated phenotype to other serotypes, by introducing through recombination via cDNA *in vitro* the genes for immunogenicity from a new strain into the genome of an ideal avirulent strain. To make such an approach feasible identification of viral genes that specify virulence is of critical importance. Therefore, the biological and biochemical characterization of several attenuated strains of different serotypes was undertaken.

A remarkable feature was a common increased electrophoretic mobility of polypeptide 3A in the attenuated strains analyzed. Sequencing data indicates a genomic deletion of approximately 60 nucleotides, depending on the attenuated strain [39]. The potential relevance of this genomic region for determining the attenuated phenotype, is being further studied by introducing an equivalent deletion in an infectious cDNA clone, recently constructed by the laboratory of Dr. Ewald Beck [40].

With regard to other infectious agents, a modified virus strain of pseudorabies virus was obtained by engineering a mutation into the thymidine kinase (TK) gene so that the activity of TK is destroyed and thus the virus cannot multiply in the central nervous system of pigs [41]. Similarly the TK gene has been deleted from infectious bovine rhinotracheitis vaccine strains which would allow a similar development of a cattle vaccine [42]. Further attenuation of pseudorabies was obtained through deletions in the internal and terminal repeat region of the pseudorabies vaccine strains [43].

One of the major disadvantages of live attenuated vaccines is that the immune response which is elicited cannot be easily distinguished from the one provoked by natural infection so that many times disease control measures are complicated. One approach to overcome the diagnostic problem is through the introduction of specific gene deletions. Such an approach was effectively used for pseudorabies. The gene coding for the glycoprotein was removed from the live vaccine, which prevents antibodies being evoked to this glycoprotein and so allows vaccinated pigs to be identified from pigs naturally infected [44].

Another way to overcome this limitation is by combining the advantages of subunit and live attenuated vaccines through the use of vaccinia vectors.

Vaccinia virus behaves as a live-virus vaccine. Therefore it stimulates cell-mediated immunity, but has the advantage that certain selected gene sequences of a heterologous agent can be inserted and expressed. Moreover, due to its large DNA capacity, it is possible to construct multivalent vaccines for different serotypes of the same virus or even against entirely different pathogenic agents. The products obtained are properly glycosilated and transported to membranes and therefore mimic the native state [45].

The large number of animal hosts for vaccinia enables its use for immunization in veterinary medicine. Other advantages of this system include:

- it is cheap to mass produce;
- it is possible to use poxviruses specific for each animal species;
- no animal reservoir is known;
- it is stable in lyophilized form at room temperature;
- it can be administered by the oral route.

Significant results were obtained with live vaccine of recombinant vaccinia virus which expresses the glycoprotein of rabies virus. Racoons fed with vaccina/rabies recombinant virus developed rabies neutralizing antibodies and were resistant to rabies challenge up to 200 days after feeding [46].

After inoculation of live recombinant vaccinia-G protein of vesicular stomatitis virus (VSV), mice were protected against lethal encephalitis and cattle protection correlated with the level of neutralizing antibody produced following vaccination [47].

More recently veterinary researches have employed vaccinia virus recombinants as experimental vaccine against Rinderpest Sindbis, Marek's disease, fowl pox and feline leukemia.

An interesting strategy was attempted for FMDV, combining the synthetic peptide

approach with recombinant vaccinia virus. However no neutralizing antibody response was elicited after inoculation into rabbits [48].

The potential hazards of human and animal virus vector vaccines should be carefully investigated before they are released for field use. The occurrence of generalized vaccinia after administration of samllpox vaccine to asymptomatic carriers of human immunodeficiency viruses (HIVs) has been reported [49].

Other interesting alternatives include:

Infectious resistant cells

Consists on blocking the production of viral proteins by inserting antisense genes (DNA fragments complementary to the viral genome) into cells. So far, attempts to use this approach have been reported for AIDS treatment and for FMD.

Anti-idiotypes

Since the observations that anti-idiotypes can mimic foreign antigens, their potential utilization in vaccines has been pursued [50]. In fact, many groups reported induction of protective immunity in experimental animals upon administration of anti-idiotypes for parasites (*Schistosoma mansonii, Trypanosoma rhodesiensi*), viruses (polio type I and II, hepatites B) and bacteria (*Streptococcus pneumoniae* and *E. coli*). Their main potential is against infectious agents which evade neutralizing by antigenic variation and for carbohydrate antigenic determinants that cannot be genetically engineered.

CONCLUDING REMARKS

At present, several new approaches to the development of animal vaccines were undertaken. Although their potential is unquestionable, an overall success will depend on the identification and expression of protective epitopes, and a deeper understanding of the molecular definitions of virulence, immunological mechanisms and molecular biology of infectious agents. Further studies are required to explain why vaccines prepared with antigens obtained by genetic engineering or peptide synthesis have been of relatively poor immunogenicity in cattle and swine when compared with antigens obtained through classical inactivated vaccines. Very encouraging is the use of new diagnostic test, which are of great importance for veterinary medicine diagnostic and particularly with regard to detection of carrier animals.

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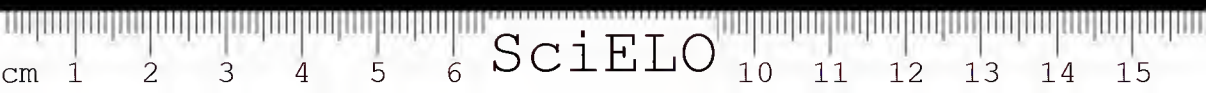
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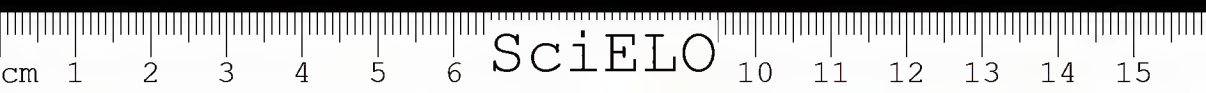
USE OF *TRYPANOSOMA CRUZI* RECOMBINANT ANTIGENS IN THE IMMUNOLOGICAL DIAGNOSIS OF CHAGAS' DISEASE.

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INTRODUCTION

Chagas' disease is still a major endemic problem in South America where some 20 million individuals display seropositivity for *Trypanosoma cruzi*, causative agent of the disease (Chagas, 1909). The life-cycle of the parasite involves two intermediary hosts (the triatomine insect and mammals) and three well-defined developmental forms, the infective non-replicative trypomastigotes, the replicative amastigotes (mammalian form) and epimastigotes (insect form) (Brener, 1973). The infection occurs when trypomastigotes, released with the excreta of the triatomine, penetrate into the mammalian host through a wound or mucosa. Alternatively, Chagas' disease can be transmitted by transfusion with infected blood. In recent years, the transmission of Chagas' disease by the triatomine invertebrate host has diminished in virtue of improvements in vector control campaigns in some of the endemic countries. However, new cases of this disease still occur due to blood transfusion with infected blood.

Transfusional Chagas' disease frequently occurs as a result of incomplete or deficient diagnosis of the disease. In a few cases, there is a complete lack of blood control and the transfusion is direct (arm-to-arm). In most cases, only one test is used for the screening of the blood, normally agglutination, which results in many false negative responses due to the poor sensibility of the test (Carrasco et al., 1985). However some

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of the blood is screened using at least two different tests. But even in this case, some false results still arise due to the low sensitivity of some methods or antigens, or cross reactivity of *T. cruzi* extracts used in the diagnosis with other diseases such as leishmaniasis, syphilis, toxoplasmosis, etc. (Camargo & Takeda, 1979).

These problems might be solved by purifying parasite specific antigens from parasite extracts. However, these antigens are not generally available and their production costs would be too high, in addition to provoking the risk of infection of people involved in the manipulation of the parasite.

On the other hand, the cloning and expression of *T. cruzi* genes in bacteria might provide antigens from the parasite with the required specificity and at low cost. Accordingly, recent work from several laboratories has resulted in the cloning and expression of *T. cruzi* genes in *E. coli* (Ibanez et al., 1988; Lafaille et al., 1989; Hoft et al., 1989; Levin et al., 1989; Cotrin et al., 1990; Paranhos et al., 1990). The results presented below describe the cloning and characterization of *T. cruzi* specific antigens and their use in the diagnosis of Chagas's disease.

RESULTS

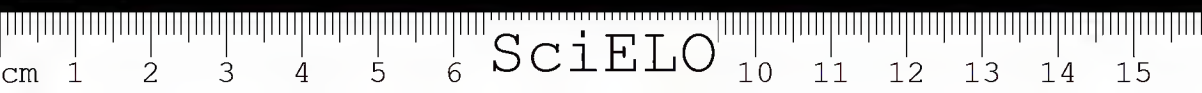
Cloning of *T. Cruzi* Antigens

The successful attempts to clone and express *T. cruzi* genes in bacteria coincided with the description of the lambda gt11 vector (Young & Davis, 1983). The general strategy consisted of the screening of *T. cruzi* expression libraries with either chagasic sera or antisera raised against the parasite. These expression libraries were either cDNA (Hoft et al., 1989; Levin et al., 1989) or genomic (Peterson et al., 1986; Dragon et al., 1987; Ibanez et al., 1988; Lafaille et al., 1989; Cotrim et al., 1990; Zingales et al., 1990), in the latter case taking advantages of the fact that the genes of the parasite are intronless.

In the work carried out in our laboratory, DNA was extracted from *T. cruzi* epimastigotes and cloned in lambda gt11 after shearing to a mean size of 1kb and subsequent addition of EcoRI linkers. The recombinant DNA library in *E. coli* was immunologically screened with a trypomastigote – specific antiserum and the positive clones were further screened with a pool of chagasic sera (Lafaille et al., 1989). Twelve clones positive with the two sera types were then purified to homogeneity.

In order to investigate the specificity of the recognition of these recombinant clones by chagasic sera, we tested the respective fusion proteins by western blot analysis against different human sera. These sera comprised both chagasic sera and sera from patients bearing diseases which cross-react antigenically with Chagas' disease. In this selection, two of the 12 recombinant clones were considered specific for Chagas' disease diagnosis.

Characterization of the Clones

The selected recombinant clones were characterized in terms of their structure and expression (Lafaille et al, 1989; Krieger et al., 1990). One of the antigens is located in the region of the flagellum adjacent to the body of the parasite, while the other is distributed in the cytoplasm of *T. cruzi*. Nucleotide sequencing analysis demonstrated that both antigens are composed of repetitive epitopes: the flagellar antigen is composed of

repetitions of a 68 aminoacid motif, while the repeat unit of the cytoplasmic antigen contains 14 aminoacids. These antigens were then named FRA (flagellar repetitive antigen) and CRA (Cytoplasmic repetitive antigen) (Lafaille et al., 1989). The consensus aminoacid sequence of the repetitive epitopes is shown in Fig. 1.

Cytoplasmic Repetitive Antigen (CRA)

LYS ALA ALA GLU ALA THR LYS VAL ALA GLU ALA GLU LYS GLN

Flagellar Repetitive Antigen (FRA)

MET	GLU	GLN	GLU	ARG	ARG	GLN	LEU	LEU	GLU	LYS	ASP	PRO	ARG	ARG
ASN	ALA	LYS	GLU	ILE	ALA	ALA	LEU	GLU	GLU	SER	MET	ASN	ALA	ARG
ALA	GLN	GLU	LEU	ALA	ARG	GLU	LYS	LYS	LEU	ALA	ASP	ARG	ALA	PHE
LEU	ASP	GLN	LYS	PRO	GLU	ARG	VAL	PRO	LEU	ALA	ADP	VAL	PRO	LEU
ASP	ASP	ASP	SER	ASP	PHE	VAL	ALA							

Figure 1 - Consensus aminoacid sequence of the repeat unit of CRA and FRA

It is interesting to note that most of the genes screened from *T. cruzi* expression libraries display various copies of repetitive motifs, indicating that repetitive epitopes are highly antigenic. Accordingly, other groups described antigens similar to CRA (Ibanez et al., 1988; Hoft et al., 1989; Levin et al., 1989) and to FRA (Ibanez et al., 1988; Levin et al., 1989; Cotrim et al., 1990). Despite the ubiquitous nature of these antigens, CRA and FRA are highly polymorphic in *T. cruzi* (Krieger et al., 1990). This polymorphis can be seen at the genomic level, where distinct restriction fingerprints are obtained for CRA and FRA in different *T. cruzi* strains.

Use Of Recombinant Antigens In Chagas' Disease Diagnosis

Although polymorphic in different strains, these antigens were recognized in the form of B-galactosidase fusion proteins by more than 95% of chagasic sera when individually tested using a radioimmunoassay procedure. This indicated that the antigenic determinants are well conserved. However, our first attempts to use these recombinant antigens in an ELISA test showed that some non-chagasic sera displayed a borderline response, very likely due to their reactivity with the B-galactosidase portion of the fusion proteins. In order to circumvent this problem, we adopted a strategy which consisted of expressing these recombinant antigens in the pMSgt11 vector (Scherf et al, 1990). This vector contains a cleavage site for factor Xa in the cloning site, hence allowing the enzymatic cleavage of the fusion protein with the subsequent release of the B-galactosidase. We first tested CRA expressed in pMSgt11 and the results were highly satisfactory: in a multi-center study carried out by the World Health Organization and involving nine laboratories and 24 antigens, it was concluded that CRA ranked as the best individual reagent (Moncayo & Luquetti, 1990).

However, a doubtful response was observed for some sera, as a result of reactivity too close to the cut-off serum control. When a mixture of CRA and FRA was used instead of the individual antigen, it became evident that some of the doubtful sera were in fact positive for Chagas' disease. Consequently, in order to improve the ELISA test we started to use a mixture (1:1) of CRA and FRA, resulting in a highly specific diagnosis reagent (Almeida et al., 1990; Krieger et al., in preparation).

The CRA+FRA ELISA was then tested using sera from different endemic regions, sera from patients bearing diseases which present cross-reactivity with Chagas' disease, and negative sera from endemic areas and from blood banks. The results were compared to those obtained with conventional serological tests (haemagglutination and indirect immunofluorescence), and to those obtained using an ELISA test consisting of a cytosolic extract (CYTO) of *T. cruzi* as antigen source. It was observed that the CRA+FRA ELISA, in addition of recognizing all tested chagasic sera, did not react with sera from patients bearing other diseases. On the contrary, both the Cyto ELISA (Table I) and the conventional serology tests (Table II) gave some false positive responses.

TABLE I

Comparision of CRA + FRA and CYTO ELISA with different human sera

Sera	CRA + FRA	CYTO
Chagasic(n=221)	221	221
Negative[1] (n=193)	0	0
Negative[2] (=49)	0	1
Schistosomiasis (n=15)	0	2
Malaria (n=12)	0	2
Syphilis (n=14)	0	4
Leishmaniasis (n=21)	0	3

1 - Negative sera from endemic areas
2 - Negative sera from blood bank

TABLE II

Comparision of the reactivity of conventional serological methods and CRA + FRA ELISA with sera which cross-react with Chagas' disease

Sera	IHA	IFI	CRA + FRA
Visceral Leishmaniasis (n=5)	5	5	0
Cutaneous leishmaniasis (n=5)	2	3	0
Leprosy (n=2)	0	2	0
Lupus (n=8)	0	2	0

CONCLUSIONS AND PERSPECTIVES

The data discussed above indicate that the use of *T. cruzi* recombinant antigens in an ELISA test provides a safe and accurate diagnosis for Chagas' disease. The main advantage of the recombinant ELISA is the fact that it gives very few (if any) false positive results in comparison to other reagents and methods frequently used for the diagnosis of the disease. These false positive responses should be avoided in virtue of the social problems they can cause for the patient. In addition, recombinant antigens are cheaper to produce than antigens isolated from the parasite, and this should have a direct impact on the price of the diagnostic reagent. In the particular case of the FRA and CRA antigens, their repetitive epitope structure suggests that synthetic peptides might be used in diagnosis in a near future. Indeed, we have recently tested a synthetic CRA peptide and the results showed that 65% of the tested chagasic sera were detected in ELISA. However, further investigations are necessary in order to determine whether this observed diminution in sensitivity was due to a technical problem related to the binding of the peptide to the ELISA plate or, alternatively, whether the problem was related to a poor exposition of the correct antigenic determinants.

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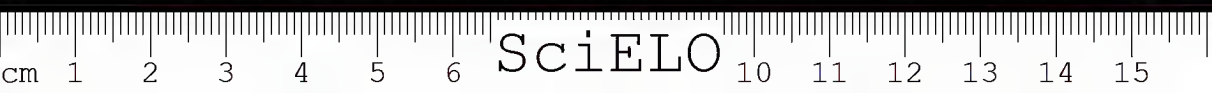
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POST-SYMPOSIUM LECTURE

PERSPECTIVES ON PRODUCTION OF GROUP B MENINGOCOCCAL VACCINES

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ABSTRACT: Group B meningococcal disease remains a problem in many countries. Since the group B *Neisseria meningitidis* polysaccharide has proven not to induce protective antibodies, existing vaccine against group B disease have been composed of lipopolysaccharide depleted outer membranes, usually in the form of small vesicles. Protection against meningococcal disease is correlated with induction of bactericidal antibodies. The group B vaccines stimulating the highest bactericidal titers when administered in a two dose immunization series 6 to 8 weeks appart consist of soluble vesicles, and one of the meningococcal polysaccharides all adsorbed to aluminum hydroxide. Efficacy trials with such vaccines have recently been conducted in Chile, Cuba, and Norway. The Cuban trial demonstrated 80% efficacy against disease caused by a B:4:P1.15 strain, and was the first to clearly demonstrate that antibodies induced to non-capsular antigens can protect against meningococcal disease.
KEY WORDS: *Neisseria meningitidis*, vaccine, outer membrane

INTRODUCTION

Serogroup B *Neisseria meningitidis* is responsible for over 80% of meningococcal diseases in Brazil and is the predominant cause of meningococcal disease in many oth-

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er countries including the United States. A recent study of serogroup prevalence in the United States[26] showed that approximately equivalent amounts of meningococcal disease were due to groups B and C. However, the incidence of meningococcal disease remains approximately 1/100,000 in the US compared to 2.5/100,000 in Brazil.

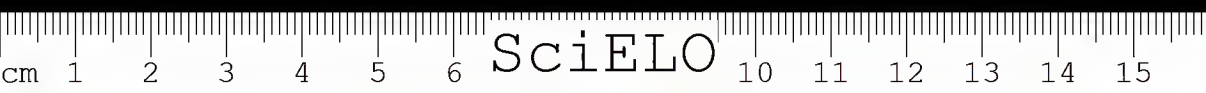
GROUP B POLYSACCHARIDE

Effective capsular polysaccharide vaccines against *N. meningitidis* serogroups A and C were developed in the early 1970s[18]. Later the Y and W135 polysaccharides were added to produce a tetravalent meningococcal vaccine[2]. Although the group B polysaccharide would be the logical choice for a group B vaccine, it is poorly immunogenic and antibodies induced by the polysaccharide do not appear to be protective. The B polysaccharide is a homopolymer of alpha 2-8 linked N-acetyl neuraminic acid, the same as on some fetal proteins. This may account for the observed poor immunogenicity of the B polysaccharide.

Attempts have been made to improve the immunogenicity of the polysaccharide [22,27,28]. Adsorption of the polysaccharide to aluminum hydroxide appeared promising in mice[28], but failed to increase immunogenicity in humans[14]. Another approach was to prepare a chemically modified polysaccharide, in which the N-acetyl groups on the polysaccharide were replaced with N-propionyl groups[21,22]. When this altered polysaccharide was chemically bound to tetanus toxoid, forming a conjugate vaccine, the N-propionyl polysaccharide induced bactericidal antibodies. Conjugates prepared using the native group B polysaccharide were nonimmunogenic. Studies are continuing by Dr. Jennings to evaluate this approach.

MENINGOCOCCAL OUTER MEMBRANE ANTIGENS

Protection against meningococcal disease is correlated with the presence of bactericidal antibodies[18]. The peak incidence of disease occurs in children under 1 year of age; as a group, they have few or no bactericidal antibodies. In addition, the high susceptibility of individuals with a deficiency of one of the terminal complement components (C5, C6, C7, or C8) for invasive meningococcal disease strongly implicates the importance of bactericidal activity in host defense against these organisms. Thus, a group B meningococcal vaccine should be based on cell surface antigens shown to induce bactericidal antibodies, although opsonic antibodies probably also contribute to protection. Studies have shown that convalescent sera from patients with group B meningococcal disease have bactericidal antibodies directed against surface proteins and the lipopolysaccharide[19,23,32]. Most efforts to develop an effective group B vaccine have, therefore, involved use of outer membrane proteins[8,41].

There is a large amount of antigenic diversity among group B meningococcal strains. The outer membrane contains three to five major proteins, comprising 5 protein classes, class 1 through class 5 having molecular weights between 26,000 and 46,000 daltons[36]. There are approximately 20 different serotypes within group B and group C based upon immunologic differences in the Class 2 and Class 3 major OMPs which are between 34K and 40K[15]. These clas 2/3 proteins are the meningococcal porins. Further antigenic diversity is seen among the approximately 46K Class 1 OMPs, which contain

the subtype specific antigens and may also have porin function. Antibodies to both the serotype and subtype proteins are bactericidal.

Although sporadic causes of group B meningococcal disease may be caused by a variety of serotypes, outbreaks and epidemics in most countries have been caused by a small number of serotypes, 2, 4, 8 and 15[1,10]. Thus, a serotype protein vaccine need contain membranes from a relatively small number of serotypes. In addition to our laboratory, laboratories in Biltoven, The Netherlands[30]; Havana, Cuba[34]; Oslo, Norway [16] and Washington DC, USA[41] have produced group B meningococcal vaccines, all based on use of lipopolysaccharide depleted outer membranes.

EARLY OUTER MEMBRANE VACCINES

Other membranes may be removed from meningococci by lithium chloride-sodium acetate extraction at 50°C. These membranes contain approximately equivalent amounts of protein and toxic lipopolysaccharide (LPS). For vaccine use, the LPS must be largely eliminated by selective solubilization with detergents, a number of which have been used for this purpose including Brij-96[13], Empigen-BB[40], and sodium deoxycholate[37]. Sodium deoxycholate may be best because it is a normal bile metabolite present in humans, and residual detergent that may be present in the vaccine would probably not have toxic effects.

To prepare OMV vaccines free of unknown quantities of group B meningococcal polysaccharide, we have isolated non-encapsulated variants or mutants using horse anti-group B polysaccharide serum incorporated into a clear agar medium to detect colonies not elaborating the B polysaccharide[13].

The first outer membrane vaccines consisted of LPS depleted membranes. The vaccine protein was separated from the detergent by ethanol precipitation and resuspended in 0.9% sodium chloride.

These vaccines were visibly particulate and contained aggregated outer membranes as observed by electron microscopy. Later studies showed that the membranes were soluble in water, but not 0.9% sodium chloride.

The early particulate vaccines as well as later vaccines contained considerable amounts of LPS (about 5 to 10 µg/100 µg protein), yet were much less pyrogenic in rabbits than would be expected[35]. The LPS that remained was strongly membrane associated, which probably accounted for the lower toxicity.

The particulate outer membrane vaccines were evaluated in adults then in children using a two or three dose immunization schedule previously evaluated in animal studies[8]. Zollinger et al.[42] found that such a vaccine failed to induce bactericidal antibodies in five adults after three doses. A similar particulate vaccine prepared in our laboratory induced low levels of antibody in both adults and children as measured by ELISA, but also failed to stimulate bactericidal antibodies. Thus, although particulate vaccines were found safe in both adults and children, they were poorly immunogenic, a fact not predicted by the animal studies.

Zollinger et al. were first to clinically evaluate soluble outer membrane vaccines[42]. They found that outer membrane vaccines could be made soluble by combination with group B meningococcal polysaccharide. Electron microscopy of similar vaccines prepared in our laboratory showed some aggregation of outer membrane vesicles (OMV) without the polysaccharide and individual vesicles with polysaccharide[13].

Meningococci release large amounts of essentially pure outer membranes during normal growth into the culture broth as blebs or vesicles[7,13]. These membranes may be purified from the broth and used as the starting material for preparation of a vaccine[13]. Since the natural orientation of the proteins in the outer membrane may be important, we have developed methods to selectively remove the LPS, leaving the membranes intact and soluble as determined by electron microscopy[13]. Soluble vaccines were prepared with and without the group B polysaccharide and tested in animals[29,37].

The soluble OMV vaccines are colloidal suspensions rather than true solutions. Increasing the ionic strength caused precipitation of the OMV, whereas addition of a negatively charged polysaccharide increased OMV solubility. The polysaccharide forms a non-covalent complex with the vesicles[43]. Recent results from our laboratory show that the OMV-polysaccharide association is hydrophobic, because removal of the lipid tail from the polysacharide by phospholipase blocked the interaction.

Soluble protein plus polysaccharide vaccines have been clinically evaluated[8,43]. These vaccines induced bactericidal antibodies on primary immunization with only modest increases in antibody titers after the second immunization. We then compared the immunogenicity of a soluble OMV vaccine with and without group B polysaccharide[8,33]. Addition of the polysaccharide resulted in a significant increase in bactericidal antibodies to a group C serotype 2a strain. The second dose 6 to 8 weeks later resulted in an increase in the percent of individuals responding to the vaccine.

The target age group for a group B vaccine is young children. When the immune responses of children were compared to those of older children and adults, by either OMV ELISA or bactericidal assay, young children (under 6 years old) responded less well[12]. In an effort to increase the percentage of young children developing bactericidal antibodies, the OMV vaccine was adsorbed onto aluminum hydroxide or[14].

Adsorption of the OMV plus polysaccharide onto aluminum hydroxide or aluminum phosphate significantly increased the bactericidal response of mice to the outer membrane proteins[37]. These vaccines were therefore evaluated in human adults[14]. The aluminum hydroxide absorbed vaccine was found safe and more immunogenic than the same vaccine without the adjuvant. The vaccine in combination with the adjuvant induced significantly higher bactericidal titers. More recent studies in Norway suggest that the adjuvant can be added directly to LPS depleted membranes[20].

In summary, these data provide evidence indicating that to stimulate an optimal immune response, the surface exposed protein epitopes need to be presented to the immune system in a near-native configuration, and therefore should remain within soluble membranes.

CURRENT VACCINES AND RECENT CLINICAL STUDIES

A study in Norway[32] using a combined serotype 2b and serotype 15 OMV vaccine showed that bactericidal antibodies were induced to both serotypes in 70% of the adults tested. This demonstrated the utility of combining multiple serotypes. The study also showed a good correlation between IgG antibodies to the outer membranes and bactericidal titers measured using human complement.

For comparative clinical studies in Norway[20] three different vaccine formulations were prepared containing OMV from a B:15:P1.16 strain mixed with either group C meningococcal polysaccharide, or with aluminum hydroxide, or with both. These vaccine

formulations were evaluated in adults and students in preparation for a large scale efficacy trial. Two doses of protein between 12.5 and 100 µg per dose were given at a 6 week interval. The researchers found equivalent bactericidal responses when the proteins were administered adsorbed to aluminum hydroxide with or without the group C polyssacharide, and that the 50 µg dose was optimal.

A group B vaccine has been produced in Cuba consisting of LPS depleted OMV from a B:4:P1.15 strain, a high molecular weight protein complex, and group C meningococcal polysaccharide, all adsorbed onto aluminum hydroxide[34]. Care is taken to control the vesicle structure and size. The vaccine contains per dose 50 µg protein, 50 µg polysaccharide, and 2 mg aluminum hydroxide. It is administered as a two dose immunization schedule with a 6 to 8 week interval. The dosage and interval were arrived at after evaluation of different immunization schedules in adults and children. Eighty-eight percent of school children responded with 2-fold or greater increases in outer membrane antibodies as measured by ELISA.

GROUP B EFFICACY TRIALS

A number of efficacy trials have now been conducted with varying results using group B meningococcal vaccines (Table 1). A number of important observations can be drawn from these trials, but the foremost is that antibodies to non-capsular surface antigens can prevent group B meningococcal disease[34]. The first trial was carried out in Cape Town, South Africa in 1981[9] against a B:2b:P1.2 epidemic (peak incidence; 150/100,000) using a 2a:P1.2 outer membrane vaccine combined with group B polysaccharide, but no adjuvant. Although insufficient cases occurred to estimate efficacy, no serotype 2 disease occurred in the vaccinated children, but equivalent amounts of disease due to other group B serotypes occurred in vaccinated and control children (received meningococcal AC vaccines). Thus the vaccine failed to protect against nonserotype 2 disease.

An efficacy trial was performed in Iquique, Chile in 1988-1990 using an outer membrane protein vaccine from a B:15:P1.3 strain combined with group C meningococcal

TABLE I

Field trials of group B meningococcal outer membrane vaccines

Years	Vaccine formulation	Location	Est. Efficacy	Ref.
1981-82	2a:P1.2 + B polysacch	Cape Town, South Africa	Too few cases	9
1987-89	4:P1.15 + C polysacch + Al(OH)3	Cuba	80%	34
1988-90	15:P1.3 + C polysacch + Al(OH)3	Iquique, Chile	51%	5
1989-91	15:P1.16 +Al(OH)3	Norway	In progress	16

polysaccharide and aluminum hydroxide[5]. During the trial over 90% of the group B meningococcal disease was due to the B:15:P1.3 clone. In this double-blind trial 40,800 volunteers, ages 1-21 years, received two doses of either the B vaccine or ACYW135 polysaccharide vaccine given 6 weeks appart. The estimated efficacy was 51%. The vaccine differed from other vaccines in that efforts were taken to reduce the LPS content to very low levels, which probably disrupted the membrane structure. The antibody responses of the children as measured by ELISA were good, but the numbers responding with bactericidal titers were low. This illustrates the need for measurement of bactericidal antibodies.

A randomized double-blinded efficacy trial was carried out in Cuba between 1987 and 1989 using the B:4:P1.15 vaccine described above[34]. The trial was conducted in 197 boarding schools, randomized by school, where there were 106,000 students between 10 and 14 years of age, half of which received the serotype 4 vaccine. During the trial 95% of group B disease was due to B:4:P1.15 and 3% to B:15:P1.15. Thus, this vaccine, like the vaccine used in Chile, was evaluated against a single group B clone. The estimated efficacy was 80%, clearly demonstrating that a protein vaccine can prevent B:4:P1.15 disease. However, since the epidemic was caused by one clone, the trial was not able to provide evidence for the degree of protection that could be expected against other group B strains.

IMPORTANT VACCINE CHARACTERISTICS AND PROBLEMS WITH CURRENT VACCINES

Immunogenicity and efficacy studies conducted with a different outer membrane vaccines have demonstrated a number of physical characteristics that are important for optimal immunogenicity of these vaccines (Table 2). A number of studies have shown that isolated outer membrane proteins induce few antibodies reactive against surface exposed epitopes. These proteins have loop structures crossing the membrane several times[25], that are not conserved upon removal from a membrane environment. We have therefore sought to maintain the vesicle structure, following detergent treatment to remove the LPS, and this is monitored by electron microscopy. Additional reasons to work with the intact membranes are that antibodies to no one protein are likely to pro-

TABLE II

Important characteristics of a meningococcal outer membrane protein vaccine

1. Must be soluble – Solubility improved by addition of polysaccharide
2. Native conformation of proteins should be maintained by: Retaining outer membrane structure OR Insertion into liposomes
3. Maintain approximately 50 μg of LPS per mg protein to retain near native outer membrane conformation
4. Outer membrane proteins normally expressed during infection should be included: Iron regulated proteins Stress proteins (heat shock)

vide broad protection against group B meningococcal disease, and we do not yet know to which proteins the critical protective antibodies are directed.

A minimum amount of LPS is required to maintain the outer membrane conformation, without which the membranes disintegrate. Isolated outer membranes contain approximately equal quantities of protein and LPS. Deoxycholate treatment removes about 95% of the LPS without disrupting the membranes, while stronger detergent treatment to remove additional LPS generally disrupts the membranes.

There are problems with all of the outer membrane protein vaccines that have received clinical evaluation to date. First, the existing vaccines are rather serotype specific. The antigenic composition of the vaccines need to be changed to provide for induction of broadly protective antibodies. In this regard, it now appears that antibodies to no single protein will provide broad protection. Most all of the outer membrane proteins appear to have antigenic variants. Second, the vaccines fail to include a number of important cell surface proteins that are expressed during infection. These in vivo proteins include the iron regulated outer membrane proteins[3,4], and probably heat-shock proteins[39]. Third, the vaccines do not appear to induce a clear booster response as would be expected of a protein antigen when the second immunization is given 6 weeks after the first. In addition, no data have been presented demonstrating whether vaccination primes for a booster response if the child is reimmunized 6 months or a year after the primary two doses immunization series. Most other protein vaccines are given as a multiple immunization series. Lastly, it may be necessary to use genetic engineering to remove the class 4 protein from the vaccine strains. These proteins are equivalent to gonococcal Protein III, and Protein III has been shown to induce blocking antibodies[31]. The class 4 protein has been removed without effecting growth properties [24].

Although vaccines consisting of LPS depleted outer membranes offer the best imediate approach, there are alternatives. An ideal meningococcal vaccine would be immunogenic in all age groups and protect against all group B strains in addition to the other disease associated serogroups, A, C, Y and W135. Development of such a vaccine will likely require a better understanding of the basic mechanisms by which only some meningococcal strains are able to gain enterance into the host and cause disease. Wetzler et al.[38] have successfully used purified outer membrane proteins inserted into liposomal membranes to induce high levels of bactericidal antibodies. Now we only need to know which proteins should be included. Another very promising finding is that alkaline detoxified meningococcal LPS remained immunogenic and induced bactericidal antibodies in mice[6].

RESUMO: A doença meningococcica continua sendo um problema em muitos países. Uma vez que o polissacáride da *Neisseria meningitidis* B mostrou-se incapaz de induzir a formação de anticorpos protetores, as vacinas existentes contra a doença meningocócica pelo grupo B tem apresentado em sua composição, membrana externa com quantidade reduzida de lipopolissacáride sob a forma de vesículas. A proteção contra a doença meningococica está correlacionada com a indução de anticorpos bactericidas. A vacina contra o grupo B estimula a produção de altos títulos de anticorpos bactericidas quando administrada em duas doses com intervalo de 6 a 8 semanas e consiste de vesículas solúveis e um polissacaride de meningococo, todos absorvidos ao hidróxido de alumínio. Testes de eficácia com tais vacinas foram recentemente feitos no Chile, Cuba e Noruega. A vacina Cubana demonstrou 80% de eficácia contra a

doença meningococcica causada por cepas B:4:P1.15, e foi a primeira a demonstrar claramente que anticorpos induzidos por antígenos não capsulares podem proteger contra a doença meningococcica.
PALAVRAS CHAVES: *Neisseria meningitidis*, vacina, membrana externa

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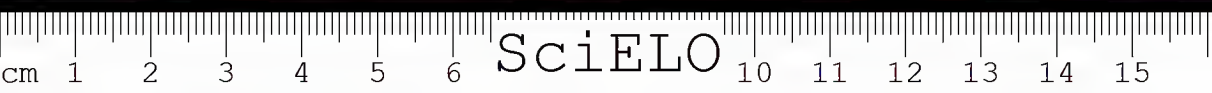
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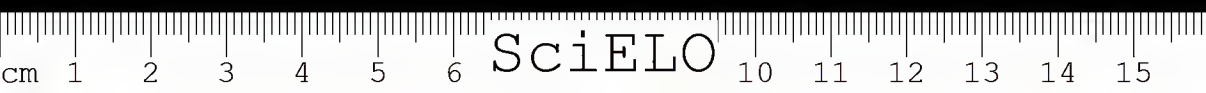
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